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A Cancerous Neoplasm of Plants

Autonomous Bacteria-Free Crown-Gall Tissue

Philip R. White, Ph.D., and Armin C. Braun, Ph.D.

(From the Department of Animal and Plant Pathology, The Rockefeller Institute for Medical Research, Princeton, N. J.)

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Plants of a great many sorts, when infected with bacteria of the species, *Phytomonas tumefaciens* (Smith and Townsend) Bergey *et al.*, develop tumorous overgrowths or "crown galls" at the point of inoculation. These are characterized by a rapid, disorganized growth involving both hypertrophy and hyperplasia of the affected cells. They are invasive, though this invasiveness is somewhat limited by the structural characteristics of plants in general. They are frequently malignant, killing their hosts by gradual disruption and destruction of the vascular system and diversion of the food supply from the adjacent uninfected tissues. They are not, however, self-limiting and when developed on perennial plants and in positions where survival of the host is possible they may reach enormous size. Smith reports crown galls on willow half a meter in diameter and weighing more than a hundred pounds (7).

On some plants, especially in the family Compositae, there are formed, in addition to the primary tumors arising at the points of inoculation, metastatic growths. These are definite tumorous processes which arise at a distance from the primary foci. They may or may not be connected to the primary foci by "tumor strands" extending out along the vascular bundles for considerable distances both upward and downward from the primary tumors. Those showing obvious strand connections Smith compared to permeation metastases in animals.[1] When the secondary tumors are without obvious strand connection to one another or to the primary foci they resemble, superficially at least, transport metastases in animals.

The primary tumors arise only upon inoculation with living cells of *Phytomonas tumefaciens,* introduced either from pure cultures grown on artificial media, in the form of extracts from infected tissues, or as masses of infected tissue transplanted from one host to another. Living bacteria can usually be isolated from primary growths, at least in their early stages, and have been assumed to be present in all developing tumors. The tumors obviously arise as a direct result of the infection and are assumed to require the presence of living, growing bacteria for their continued development. Although possessing the superficial characteristics of cancerous growths they are, therefore, apparently not truly autonomous.

[1] Most of the papers dealing with past work on this subject have today, so far as the medical reader is concerned, little more than an historic interest. The authors have chosen to keep their bibliography to a minimum. The reader is referred to Riker and Berge (5) and White (9–11) for information sufficient to permit a general orientation.

Demonstration of bacteria in tumor strands has regularly proved much more difficult than in the case of the primary tumors. This is doubly true of the secondary tumors which Smith found to be generally bacteria-free (8), an observation which one of us has confirmed (1, 2).

It is the purpose of this paper to present evidence that these secondary tumors are not only characteristically bacteria-free but that they possess, in the absence of any detectable tumor-inducing agent, a unique capacity for unlimited, disorganized, autonomous growth *in vitro* as well as *in vivo.* Since this renders them capable of producing new typical crown-gall tumors upon reimplantation into uninfected hosts of the same or closely related species, these tissues provide a very close analogy in all fundamental respects to animal cancers.

METHODS

Seeds of *Helianthus annuus* L. were planted in 6-inch pots of soil and allowed to germinate. When the plants were about 3 weeks old, bacteria from 48-hour nutrient dextrose agar cultures of the highly virulent A_6 strain of *Phytomonas tumefaciens* were injected by needle puncture into the plants at a point at least 3 inches from the apical bud, in this case just above the cotyledons. Primary tumors arose regularly at the points of inoculation. Six weeks to a month after inoculation, secondary tumors of the type described elsewhere (1) began to appear on the petioles and midribs and at the nodal regions, at considerable distances above the primary tumor, on from 30 to 50 per cent of the infected plants. When the nodal tumors had reached the size of a small pea, they were extirpated and divided into aliquots. Tissue from the interior of one group was tested for bacterial content. Tissue from the other was removed aseptically and fragments were planted on an agar nutrient

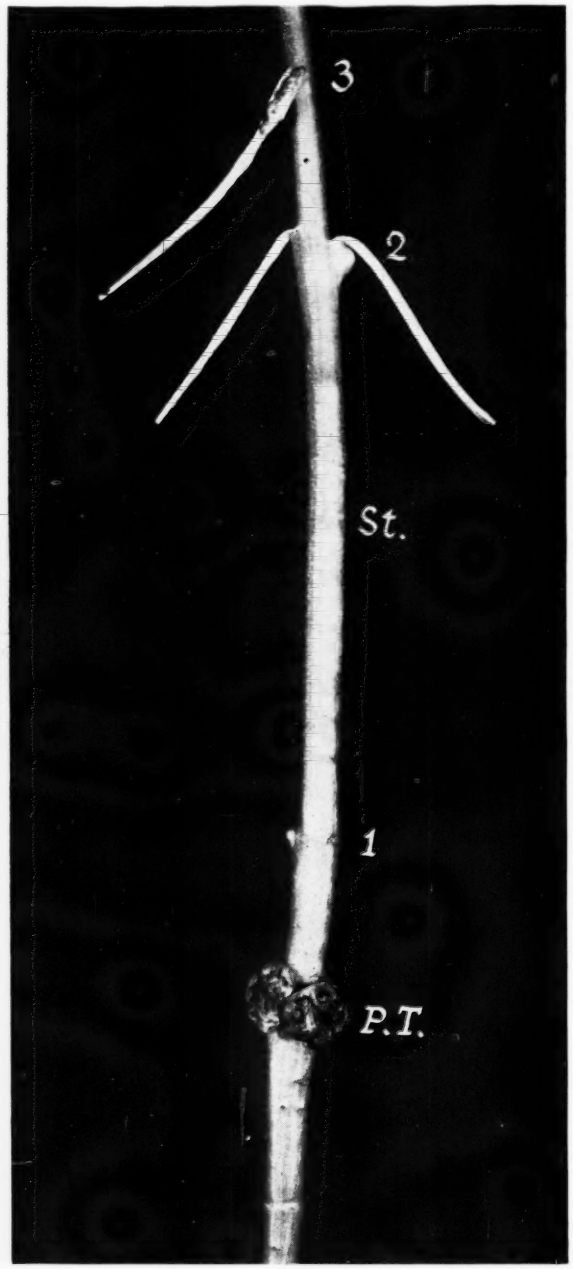

FIG. 1.—A sunflower plant 6 weeks after inoculation just above the cotyledons with a virulent strain of *Phytomonas tumefaciens*. Inoculation was by a single puncture with a needle dipped in a broth suspension of crown-gall bacteria. *P.T.*, primary tumor developed at the point of inoculation. *1*, leaf scar at the 1st node above the primary tumor bearing a small nodular secondary tumor. Crown-gall bacteria are usually present in such tumors. *St.*, a series of unerupted tumors adjacent to the vascular bundles for some distance above the primary tumor and sometimes connected by tumor strands. Crown-gall bacteria can sometimes be isolated from these regions. *2*, leaf bases representing the 2nd and 3rd nodes above the primary tumor. One bears at its base a well developed, smooth secondary tumor. Crown-gall bacteria can but rarely be isolated from such tumors. *3*, leaf base at the 4th node above the primary tumor. The petiole carries a series of erupted and seminecrotic secondary tumors. Such tumors seldom yield cultures of crown-gall bacteria but frequently contain air-borne and water-borne contaminants of various kinds.

adapted to the growth of tissue cultures. Cultures which showed any bacterial or fungous infection in the 1st passage or which failed to grow were discarded. Those that were retained were divided and transplanted at 2-week intervals. At the end of 5, 10, 15, 20, 25, and 30 passages, aliquots of cultures were subjected to a variety of tests. Some were ground in nutrient broth or agar to determine their possible bacterial content, or were transplanted into the stems of healthy sunflower or artichoke plants to determine their capacity to produce tumors. To determine whether a tumor-inducing virus was present, others were ground to a mass free of living sunflower cells and inoculated into healthy sunflower plants. Still others were used in inoculating animals preparatory to a serological test for detection of the possible presence of noncultivable bacteria.

RESULTS

The development and sterility of secondary tumors.[2] —Two distinct types of secondary tumefaction have been described in sunflower plants inoculated with the crown-gall organism (1). The first, found in the petioles and often extending into the larger veins of the leaves, consisted of small shallow swellings and strands. The second type, which was more nodular in character, occurred at or just below the insertion of the leaves. Both types were found as far distant as 4 nodes above the point of inoculation.

Attempts to isolate bacteria from tissues of these tumors were carried out as follows: The epidermis

[2] The idea has become firmly established, and is frequently stated in the literature, that *Phytomonas tumefaciens* is a difficult organism to isolate and grow. This idea apparently has arisen from the fact that difficulty has often been encountered in isolating the organism from a number of foci, especially old tumors, secondary tumors, and tumor strands. It has been *assumed* that no tumor growth could proceed without bacteria being present, and failure to isolate organisms from such tissues has led to the conclusion that the technic must be faulty and the bacteria refractory in nature. The alternative possibility, that tumorous growth once initiated may sometimes proceed in the absence of bacteria, has been ignored. Actually, those who have worked with *Phytomonas tumefaciens* know that there is no difficulty whatever in reisolating these organisms from any tissue or suitable medium into which they have recently been placed and in growing them on a variety of media. One of us (A. C. B.) has repeatedly recovered viable cultures from preparations known to contain as few as 10 bacteria (unpublished experiments). The data presented in the present paper will serve to demonstrate that typical tumors may develop in the complete absence of bacteria. We therefore believe that this idea of the refractory nature of *Phytomonas tumefaciens* has been based on a misconception in the minds of most workers in the field and is actually without real basis in fact.

was peeled from the affected region, exposing a sterile surface of tumor tissue. No disinfectant was used. Fragments of tissue were dissected out under aseptic conditions and placed in sterile Petri plates containing each 1 ml. of nutrient dextrose broth. The tissue was then pulled apart by means of sterile dissecting needles and permitted to incubate in the broth for periods ranging from 1 to 8 hours. Nutrient dextrose agar, potato dextrose agar, yeast-water mannitol agar, and Patel's medium (4) were used for pouring the plates. They were incubated at 25° C. and were not pronounced negative until they had been under observation for at least 3 weeks.

A total of 216 secondary tumors was examined. These fell into 2 groups. Of 35 tumors developed at the first node above the point of inoculation, that is within about 10 cm. of this point, 29 (83 per cent) were found to contain the crown-gall organism. The technic is, therefore, effective in isolating these organisms from tissues known to contain them. On the other hand, of 181 tests on tumors found at distances greater than 10 cm. above the primary tumor, only 7 (3.9 per cent) were infected with the crown-gall organism. The bacteria found in the latter group were present in very small numbers. Most plates showed only a single colony. The possibility exists that other supposedly sterile tumors may also have contained crown-gall organisms in extremely small numbers but in such condition that they were not able to initiate growth on the media used for isolation purposes. It seems more probable, however, that those occasionally found may have traveled up the xylem vessels and may not have been present free in the tumor tissues at all. Stapp, Müller, and Dame were able to isolate *Phytomonas tumefaciens* from apparently healthy tissues of *Datura tatula* L. at a distance

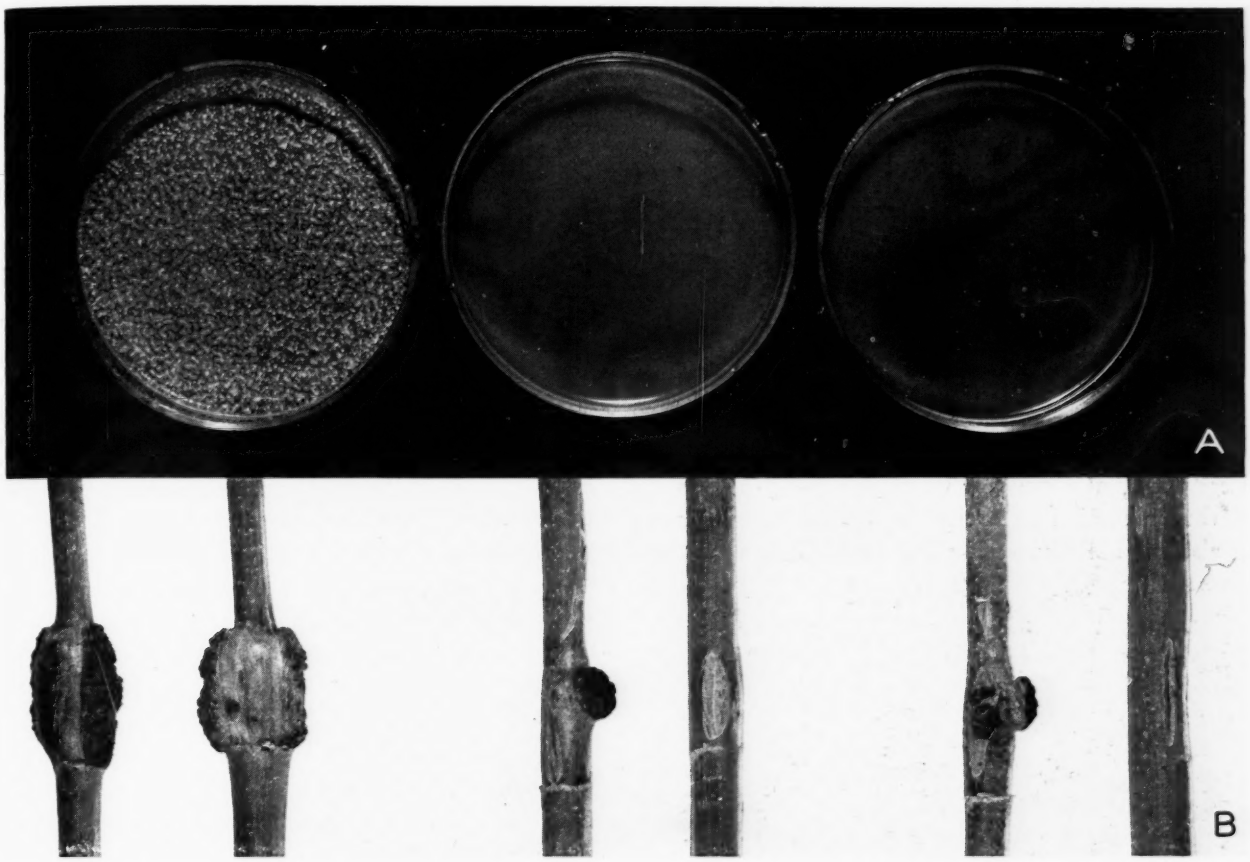

FIG. 2.—(A) Petri plates 72 hours after inoculation with triturated tissue from (reading from left to right) a primary crown-gall tumor, a secondary crown-gall tumor 2 nodes above the primary, and a tissue culture derived from a secondary tumor and maintained for 21 passages *in vitro* on a synthetic nutrient. Dark spots are tissue debris. The plate at the left shows thousands of colonies of *Phytomonas tumefaciens,* while those at the center and right are entirely sterile. (B) Sunflower plants inoculated with material corresponding in origin to that tested bacteriologically in the series of plates shown above. The left-hand member of each pair was inoculated with a fragment of tissue which was excised but not triturated, so that it contained viable tissue cells, while the right-hand member was inoculated with the corresponding tissue which had been ground so as to destroy the viability of the tissue cells but to release any bacteria occluded therein. With tissue of the primary tumor (left-hand pair) known to contain bacteria, trituration does not diminish the tumor-forming capacity of the inoculum. With tissue from secondary tumors and from tissue cultures, on the other hand, tumors are produced only when viable tumor cells are present.

of more than 100 cm. above and below the primary tumor. Other workers have reported similar results in a variety of host plants. One of us has isolated the crown-gall organism in a few instances from apparently healthy sunflower tissue at distances of 30 cm. or more from the primary tumor. Whether the bacteria, when confined to the vessels, are capable of bringing about cellular disturbances in their vicinity is not known with certainty.

The evidence is clear that secondary tumors separated by distances of more than one internode from the primary focus are characteristically free of crown-gall organisms. This is in agreement with Smith's data (8), though not with his interpretation (2).

Isolation of tissue cultures from secondary tumors.— The technic for preparing tissue cultures was similar to that used in isolating tissues for bacteriological tests. The epidermis was stripped aseptically from the surface of the tumor or, in case of large tumors, the tumor itself was broken free of the subjacent tissues exposing an aseptic surface. Fragments of tissue a millimeter or more in diameter were excised from the exposed regions with a surgeon's ear curette. These were planted on a nutrient previously developed for the cultivation of excised tissues of other plants (9, also unpublished results). Its constitution was as follows:—

$MgSO_4 \cdot 7H_2O$	360	mgm.
$Ca(NO_3)_2$	200	"
Na_2SO_4	200	"
KNO_3	80	"
KCl	65	"
NaH_2PO_4	16.5	"
$MnSO_4$	4.5	"
$Fe_2(SO_4)_3$	2.5	"
$ZnSO_4$	1.5	"
H_3BO_3	1.5	"
KI	0.75	"
Glycine	3.0	"
Thiamin	0.1	"
Sucrose	20	gm.
Agar	6.0	"
H_2O	1,000	ml.

Cultures were made either in 25 × 150 mm. pyrex tubes containing 15 ml. of nutrient sloped to expose an extensive surface, or in 125 ml. pyrex Erlenmeyer flasks containing 50 ml. of nutrient. The nutrient was sterilized in the culture vessels by autoclaving for 20 minutes at about 1 atmosphere imposed pressure. This nutrient supports a rapid growth of a variety of plant tissues including roots of many species of dicotyledons, callus tissues of *Nicotiana,* etc. It has also been shown to support a profuse growth of *Phytomonas tumefaciens* and thus serves as a check on the possible occurrence of this organism in any tissues implanted therein.

Isolation of sterile tissues from the interior of petiolar tumors proved difficult. The pieces of tissue were of necessity very small and, because of the high proportion of woody tissues in these regions, they were difficult to cut, rendering the chances of crushing or other mechanical injury high. Of a preliminary series consisting of 41 fragments of tissue from such tumefactions (Nov. 29, 1940), 3 were contaminated with molds, 11 were obviously killed by the operation, 16 were in doubtful condition, and 14 made some growth. Only 2 of these showed any considerable growth even at the beginning, and they for unknown reasons ceased to grow after about a month. Of a 2nd series of 40 cultures (Mar. 11, 1941), 5 were contaminated and only 3 were still growing at the end of a month. The petiolar tumors were thus not satisfactory material with which to work. The round nodal tumors proved much more satisfactory. Tumors about the size of a pea were carefully broken free of the host. The broken surface presented a sterile region through which fragments of parenchymatous tissue could be removed from the heart of the mass without the operating knife or curette coming in contact with the epidermis which had been exposed to possible air-borne or water-borne contamination. In a preliminary series of 17 cultures isolated from 3 tumors (Feb. 10, 1941), there were no contaminations and 9 cultures grew. One of these, No. 7, constitutes the starting point for the material upon which most of the present study is based. In another series of 20 cultures from 4 tumors (Mar. 25, 1941) a single one was contaminated and 11 grew.

To establish further the possibility of isolating successful tissue cultures from nodal secondary tumors on sunflower, a series of seedlings was inoculated with *Phytomonas tumefaciens* on May 20, 1941. On July 24, 22 stems, each bearing one or more secondary tumors, were cut and photographed. On the following day 62 isolations of tumor tissue were made from 12 of these tumors. Some had already ruptured the epidermis rendering the chance of finding secondary invaders high. Out of these 62 isolations, 6 were contaminated with molds and 22 with miscellaneous bacteria. All isolations (20 in number) from tumors No. 2B, 3, 5, and 6 were contaminated. All cultures (likewise 20 in number) from tumors No. 1, 2A, 20, and 22B were sterile. Scattered contaminations occurred in tumors No. 4, 8, 10, and 22A. The bacterial colonies isolated from these cultures appeared for the most part quite different from colonies of *Phytomonas tumefaciens* but, as a further check, they were first grown in bouillon and then injected into sunflower, tobacco, or tomato plants to determine their

pathogenicity. In no case did they produce tumors.[3] All therefore represented secondary invaders or contaminants in no way connected with the crown-gall organisms originally introduced elsewhere in the plants. Tissue cultures from the uncontaminated tumors were maintained for a minimum of 12 passages *in vitro*. Those isolated from different tumors showed quite different growth rates, and only those from Nos. 1, 4, 8, 10, and 22B can be considered really satisfactory as sources of tissue cultures. These 5 strains have been retained and are now (May, 1942) in the 20th passage. The strain isolated from No. 10 grew especially rapidly and consistently. This difference in growth rates suggests that the capacity for autonomous growth may be a quality which is acquired gradually. It seems probable that a study of these early stages in the development of autonomy may give us valuable information as to the real nature of the processes involved.

It can be unequivocally stated, as a result of the experiments presented above, that inoculation of sunflower seedlings with *Phytomonas tumefaciens* regularly results in the formation of primary tumors; in a considerable percentage of cases there are formed secondary or metastatic tumors; these secondary tumors are with rare exceptions free of the original inciting organism, though sometimes infected with air-borne or water-borne contaminants; and tissues of these tumors can in many cases be successfully established in tissue cultures as rapidly growing, bacteria-free masses. A detailed description of these cultures will be given later.

Isolation of tissue cultures from healthy tissues.—In order to have healthy material for comparison with the cultures of tumor tissues, meristematic tissues from various regions of uninfected sunflower plants were examined in a series of 40 cultures set up on Apr. 17, 1941. Of these 10 were taken as entire transverse segments of procambial region after stripping off the epidermis, 10 from the internodes of old woody stems so as to include the cambium, 10 from the nodal region of old stems so as to include cambium, 5 from the petiole of a mature leaf, and 5 from the petiole of a very young leaf. Four of the 40 cultures became contaminated. Growth occurred in some isolations from all regions except the petiole of the mature leaf. Those from the petiole of the young leaf grew very poorly. The segments of internodal tissue from old stems showed extensive proliferation only at the cambial level, while those from the procambial region and from the node showed areas of proliferation scattered over the entire cut surface. Cultures of these healthy tissues were carried through a minimum of 15 passages.

Isolation of tissue cultures from noninfectious tumors.—Tumor-like growths are sometimes produced in nature by various irritants, such as the stings of certain hymenopterous insects. They can also be induced artificially by a variety of chemical treatments, such as prolonged contact with any of a number of indole compounds and related substances. These tumors induced by chemical treatment have been observed to continue development only so long as the irritant is maintained in contact with the host tissues. The same is presumed to be the case with insect galls. Since one interpretation of the origin of metastatic crown-gall tumors involves the supposed movement of "growth substances" comparable to these chemical irritants from the primary focus of infection through the host tissues to new foci, it was thought desirable to compare the behavior of tissues taken from tumors known to have been produced by such substances with that of secondary crown-gall tissues. For this purpose, young plants of sunflower were sprayed (Mar. 12, 1941) with a heavy suspension of indole-oxalo-acetic acid (12). Isolations of tissue were made on June 7 from galls developed on 2 plants so treated. Of 20 isolations, 7 grew. Cultures therefrom were maintained for 19 passages.

Characteristics of tissue cultures of tumorous and nontumorous origin.—The fragments of tissue isolated for cultivation, whatever their origin, are regularly characterized by the flat surfaces left by the operator's knife. Cultures which fail to grow retain these flat surfaces, while in those which do grow the surfaces are distorted over greater or less extensive areas and with greater or less rapidity depending on the extent and activity of the proliferation which occurs. This forms a visual criterion for the first stages of growth. Cultures of petiolar tumefactions rarely showed any proliferation and then only in the form of isolated papillae. Segments of internodal tissue from old uninfected stems showed more extensive proliferation, but from the cambial region only. Cultures from the procambial region of such stems grew chiefly from the medulla so that their outlines became those of rounded cushions. Cultures from all tumors grew more nearly alike from all surfaces, so that they gradually took on a globular outline.

Tissue cultures from uninfected plants retained their tendency to grow from only restricted areas for many passages and usually so long as they continued to grow at all. This tendency may be interpreted as a polar response. As a result of this unidirectional

[3] Hildebrand (A Micrurgical Study of Crown Gall Infection in Tomato. J. Agr. Research, **65**:45-59. 1942) has shown that typical crown galls may be produced with considerable regularity by inoculating tomato or tobacco plants with single bacteria and that inoculation with preparations containing 50 or more bacteria can be relied on to produce galls.

growth and of the slow proliferation, the cultures usually showed considerable areas of darkened, old, and sometimes necrotic remnants of the original explant accompanied by smooth-surfaced compact masses of growing tissue. They often differentiated well organized vascular elements and became fibrous and woody. This tendency to a high degree of differentiation was accompanied by the formation of considerable amounts of chlorophyll and the frequent formation of roots. These healthy-tissue cultures were, therefore, not essentially different in behavior from any cuttings made as very small fragments. They retained a considerable degree of subservience to the morphogenetic restraints which characterize normal tissues within the intact plant. This unilateral, compact, green, woody, and sometimes rooted, type of development was characteristic of cultures taken from chemically induced tumors as well as those from healthy plants.

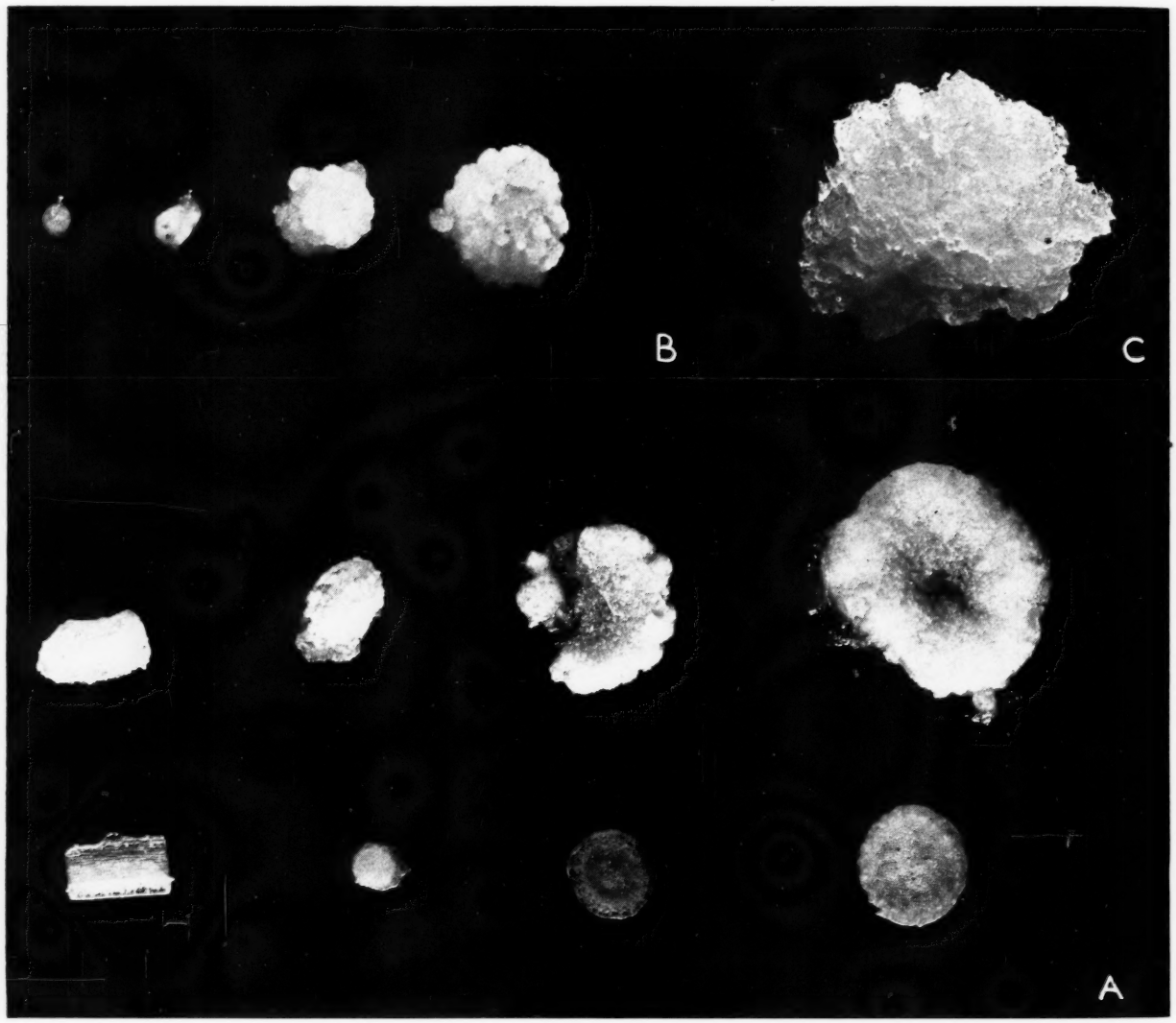

FIG. 3.—(A) Lower row, tissue fragments excised for cultivation from a healthy sunflower plant. Reading from left to right: *1*, a small prismatic sector from a mature internode containing pith (above), vascular tissue (center), and cortical parenchyma (below); *2*, a cross segment from the procambial region; *3*, a cross segment from a young node; and *4*, a cross segment from the center of an older node. Upper row, first-passage tissue cultures from pieces corresponding in form and origin to those shown below, 2 weeks after excision. Note the dense, smooth character of the overgrowth. Mag. × 2. (B) At the left, a fragment of tissue excised from a secondary crown-gall tumor. At the center and right are 3 stages in the development of tissue cultures from such a fragment. Note the difference in surface texture between these tumor tissue cultures and the healthy tissue cultures shown in A. Mag. × 2. (C) A tumor-tissue culture at somewhat higher magnification. Mag. × 5.

These cultures grew very slowly. Those from healthy tissues increased about 250-fold in a year's time. Tissue cultures from indole-oxalo-acetic acid tumors increased only about 50 times in the same period. Their degree of autonomy, as expressed in a low growth rate and in a high degree of morphogenetic conformity, was thus quite feeble.

Tissue cultures from secondary tumors presented

a notable contrast to these characteristics. They tended to grow from all surfaces alike, though not all at equal rates, so that the surfaces became covered with innumerable irregular protuberances which were constantly changing as growth progressed. Growth was very rapid, so that much material had to be discarded at the close of each passage. On the assumption that all discarded cultures were capable of growing at rates equal to those observed in the cultures retained, the clone started from culture No. 7 (mentioned above) grew at a rate giving a theoretical increase during 30 passages of about 4.4×10^{14} or a hundred million million times. This rapid growth left little time for differentiation. The cultures were regularly glistening white in color except for occasional scattered necrotic cells. They were spongy, open, and friable in texture. Although scattered vascular elements were formed, these were so irregularly distributed that they did not impart a fibrous or woody texture to the cultures even when these attained considerable size. The cultures never developed roots. The accompanying figures show clearly the appearance of cultures from both healthy and tumorous tissues. The entire behavior of tumor tissues is one of marked autonomy, of almost complete escape from normal morphogenetic restraint. The tissues have retained *in vitro* and have

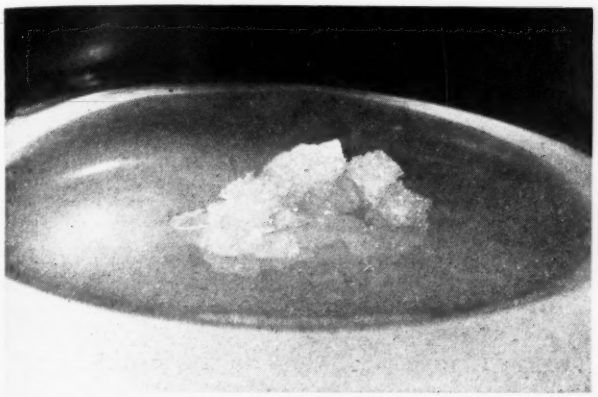

FIG. 4.—A tissue culture of secondary tumor tissue from sunflower maintained for five 2-week passages *in vitro* and then left on nutrient agar for 6 weeks without transfer. Although this nutrient is capable of supporting a profuse growth of crown-gall bacteria, none have appeared though the increment of the tissue has been many fold. Mag. × 1.4.

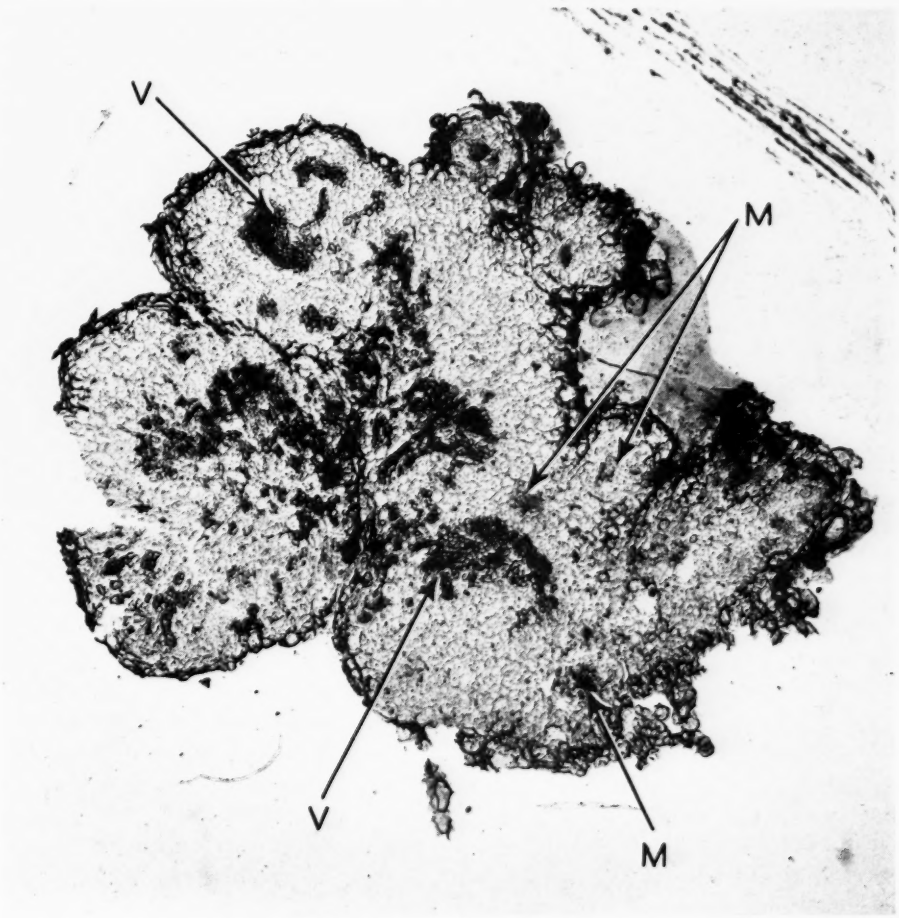

FIG. 5.—Transverse section of a tissue culture similar to that shown in Fig. 3A, right. The lobed, mostly parenchymatous outer portion contains scattered meristematic areas (*M*) and has a surface covered with enormously enlarged callus cells. In the center are scattered groups of scalariform cells (*V*). These are obviously not organized into any coherent conducting system. Mag. × 23.

even expressed in exaggerated degree the rapid disorganized growth which characterizes them within the attached tumors and which differentiates them both *in vitro* and *in vivo* from healthy tissues.

Bacterial sterility of tumor tissues grown in vitro.—The clone of cultures derived from isolation No. 7, set up on Feb. 10, 1941, may be taken as typical of tumor tissue cultures from sunflower. The culture developed from this fragment was divided on Feb. 25 into 5 pieces. On Mar. 20, 2 of these, which were growing rapidly, were divided into 6 pieces. On Apr. 8 these 6 pieces were divided again to make 19 and, on Apr. 22, the first 10 were divided to make 25 cultures. The last 9, although in good condition and growing well, were discarded. Thereafter, a stock of 25 was maintained and the cultures were regularly transferred at 2-week intervals. At the beginning of the 10th, 15th, 20th, 25th, and 30th passages the number was increased to 100, and all cultures were tested at the end of each of the first 4 of these periods for bacterial content, for tumor-forming capacity, and for possible virus content. Additional bacteriological tests were made on occasional cultures taken from passages other than these 4.

Tests for bacteria were made in several media known to favor the growth of the crown-gall organism. On July 18, 1941, 100 cultures (passage 10) were crushed separately in petri dishes containing nutrient dextrose broth. These were incubated for from 1 to 8 hours. Plates 1 to 24 were then poured with potato dextrose agar, plates 26 to 100 with nutrient dextrose agar. Eighteen plates were contaminated with either bacteria or molds, mostly in single colonies and having an appearance clearly differentiating them from *Phytomonas tumefaciens*. All bacterial colonies were inoculated into young tomato plants. In no case did tumors develop. On Sept. 26, 1941, 100 cultures (passage 15) were again tested. The tissues were crushed in nutrient dextrose broth and incubated for several hours. Plates 1 to 33 were then poured in nutrient dextrose agar, Nos. 34 to 66 in potato dextrose agar, and Nos. 67 to 100 in Patel's agar (4). Two cultures were contaminated with molds and 9 with bacteria. The 9 bacterial colonies were inoculated into tomato plants

FIG. 6.—Detail from the central vascularized region of Fig. 5, showing the scattered and disoriented scalariform cells interspersed with unmodified parenchyma. Mag. × 250.

but no tumors resulted. On Dec. 2, 1941, a 3rd set of 100 cultures (passage 20) was tested in like manner. Of these, 6 showed mold contaminations and only 3 bacteria. None of the bacteria produced galls on inoculation into tomato plants. On Feb. 27, 1942, a 4th set of 100 cultures (passage 25) was tested. Of these, 6 showed mold contaminations. No bacteria appeared on any of the plates.

Similar tests were made on a series of about 300 cultures maintained as a stock of material for various physiological experiments and mostly derived from passage 13 of this clone. In no case was *Phytomonas tumefaciens* recovered.

As a further check on the presence of crown-gall bacteria in these tissues, the tissue cultures were subjected to serological tests. Rabbits weighing slightly more than 2,000 gm. were used. All rabbits were bled at the beginning of the experiment and their sera tested for agglutinins for the A_6 strain of *Phytomonas tumefaciens* which was used in this work. All were negative. Two rabbits were injected at spaced intervals with antigen prepared from cultures of tumor tissues which had been maintained *in vitro* for 15 or more passages. As checks, 2 additional rabbits were injected with antigen prepared from tissues of primary tumor about 1 month old and known to contain the crown-gall organism in considerable numbers. The antigens were prepared by thoroughly grinding 3 gm. of the tissue in question with sterile saline (0.85 per cent NaCl) in a mortar. The suspensions were filtered through a 60-mesh wire sieve and injected intraperitoneally in 5 ml. doses except for the first injection, in which 2 ml. were used. Twelve injections were given at 4-day intervals. Trial bleedings revealed that the maximum titer against the crown-gall organism in the bacteria-containing tumor tissue was reached after the 9th injection and no increase in titer was observed during the last 3 injections. A saline suspension of a 36-hour nutrient dextrose agar culture of the A_6 strain of the crown-gall organism was used as the test antigen.

Agglutination tests showed that the sera of the 2 rabbits injected with bacteria-containing primary tumor tissue had titers against the A_6 strain of *Phytomonas tumefaciens* of 1:180 and 1:320, respectively, while the other 2 sera prepared by injecting tissue cultures of secondary tumors into rabbits reacted negatively against the A_6 strain even at serum dilutions as low as 1:1. Complement fixation tests in which formolized bacillary suspensions of the A_6 strain were used as test antigens fully confirmed the results of the agglutination tests. These results support the conclusion that the tissues obtained from secondary tumors were free of crown-gall bacteria.

Of the approximately 700 cultures tested bacteriologically in these series not a single one contained *Phytomonas tumefaciens,* the organism originally responsible for the formation of the tumors from which the tissues were isolated. Detailed observations were made on 1,056 cultures of this clone, and many hundreds more were grown as experimental material for serological, enzymatic, histological, and other studies. All were maintained for periods of 2 weeks or more, growing in intimate contact both at freshly cut and at intact surfaces with a nutrient shown to be capable of supporting a profuse growth of *Phytomonas tumefaciens,* without ever producing colonies of this organism. Serological tests likewise failed to disclose the presence of such organisms. All this evidence provides, we believe, unequivocal ground for believing these tissues, as maintained in tissue culture, to be entirely devoid of the crown-gall organism.

Virus sterility of tumor tissues grown in vitro.— Triturated tissues of primary bacterial galls, when injected into tomato or sunflower plants, regularly produce rapidly growing tumors, while similarly prepared tissues of healthy plants produce, on injection, no more reaction than that attributable to their content of growth substances and "wound hormones." Ground tissues of this sort are regularly used as a source of certain plant disease viruses, especially those of the tobacco mosaic group. If the difference in behavior between bacteria-free tumor tissue and healthy tissue were due to a virus one might expect, though obviously without complete certainty, that this virus might be carried over in tissue pulp. To test this, 100 cultures (passage 10, July 15, 1941) were separately ground to a paste on sterile glass slides, using sterile glass spatulas. The debris of each was inserted into a 1-month-old sunflower plant at the cambial level. The wound was then closed tightly with sterilastic tape. At the end of 2 months, 99 grafts had healed without any significant deviation from normal wound reaction. One graft, No. 63, had developed a small, smooth tumor about 3 mm. in diameter. Tests made on Patel's medium showed this tumefaction to be free of bacteria. It probably arose from a few cells of the culture which may have remained uninjured and viable (as described later) even after the grinding to which the tissue had been subjected. In a second similar series, set up Sept. 25, 1941, not a single graft showed any significant deviation from normal wound reaction. The evidence obtained from these 200 tests serves, first, to corroborate the previous evidence that these cultures were free of *Phytomonas tumefaciens* and, second, to demonstrate that, *if* the abnormal proliferative activity of these tissues was due to the presence of a virus, this could not be of the stable, easily transmissible sort with which we have to deal in the tobacco mosaic

group and in the chicken sarcoma I. While this does not preclude the possibility of a virus of different behavior having been involved, there is no evidence to suggest the existence of such a virus, and it would surely be superfluous to postulate such a factor in the absence of any evidence.

It is clear, therefore, that these tissues, derived from secondary metastatic tumors induced in young sunflower plants by *Phytomonas tumefaciens* present elsewhere in the plant but not demonstrable at the site of the secondary tumors, were not themselves carriers of bacteria and were capable of unlimited growth *in vitro* at rates greatly in excess of, and in habit quite distinct from, those of similar cultures from plants not infected with crown gall. It is clear that, as Jensen said of the tissues of old crown galls of sugar beets with which he was dealing (3), "under the influence of the bacteria" (in this case, an influence apparently separated by some distance from the bacteria themselves) "the host cells acquire an increased independence, taking on the character of parasitic cells in a manner similar to that observed with carcinoma and sarcoma cells" (". . . . at Cellerne under Bakteriens Paavirkning aendres saaledes, at de selv opnaar større Selvstaendighed og antager Karakteren af 'snyltende Celler' paa lignende Maade, som Tilfaeldet er med Karcinomets og Sarkomets Celler"—pp. 130-131). These experiments tend to confirm Jensen's conclusion that plant cells may, under some circumstances, undergo a radical change in their growth behavior similar to that supposed to occur in the development of spontaneous malignant growths in animals, resulting in a marked increase in autonomy and a corresponding escape from normal morphogenetic restraints.

The tumor-forming capacity of bacteria-free tumor cells.—The capacity which these bacteria-free tissues show of overriding the morphogenetic restraints controlling the development of unaffected tissues, even *in vitro,* suggested that this independence, which is a characteristic of tumor tissues *in vivo,* might be capable of expressing itself in new hosts by the production of new tumors. This possibility was confirmed in a series of tests.

In a preliminary series, tissue from 5 cultures derived from 2 metastatic tumors was grafted in a variety of ways into young sunflower plants (May 6, 1941). Extensive growth of the implant depends, of course, as it does in animal experimentation, on early and satisfactory vascularization of the growing mass, so that takes can be expected only if the implants are so placed that vascularization can occur. Implants in the pith, within the confines of the vascular ring or in the cortex outside of this ring, cannot be expected to take with any considerable frequency. A high percentage of failures is therefore to be expected. All cultures used in this first series had been maintained *in vitro* for 5 consecutive passages. Six weeks after implantation one host plant had been broken accidentally and discarded, 4 had healed without reaction other than that normal to wound healing, while 5 (50 per cent) had formed typical crown-gall tumors. One tumor was tested

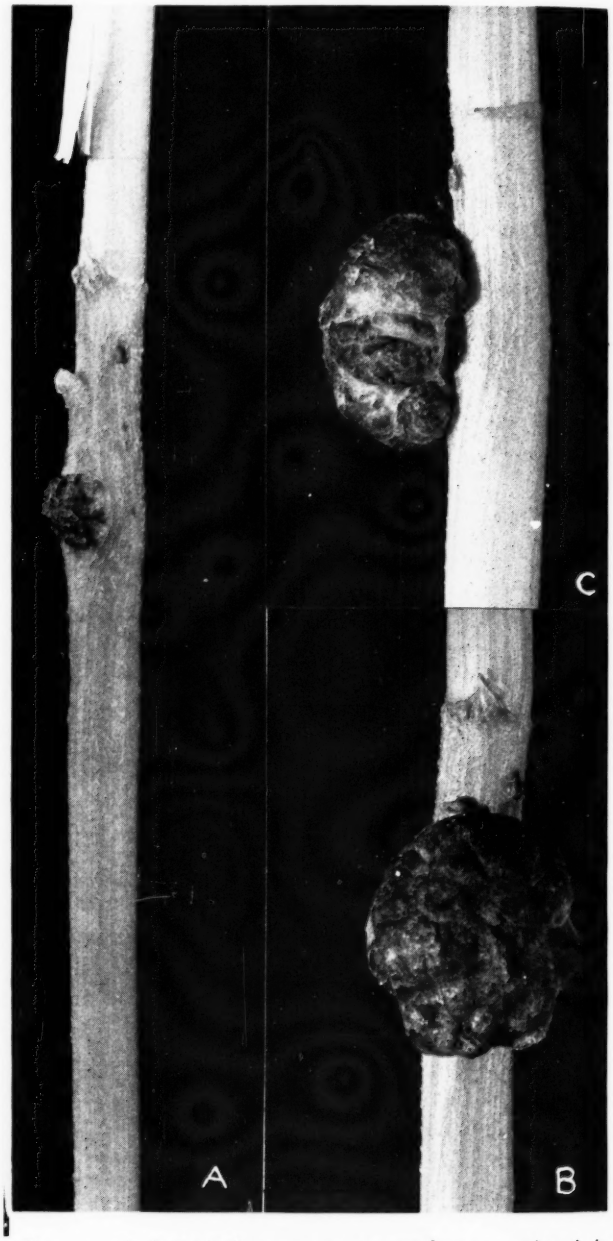

FIG. 7.—A bacteria-free tumor on sunflower produced by ingrafting a fragment of tumor-tissue culture (passage 10) similar to those shown in Fig. 3. (A) One month after inoculation. (B and C) Two months after inoculation (B, face view; C, profile). Note the stub of tissue above the tumor, a remnant of the flap of cortex under which the implant was placed. The tumor has developed only at the site of the graft, while the remaining extent of the wound has healed normally. Mag. × 1.5.

for bacteria and found to be bacteria-free. Tissue cultures were reisolated from another tumor and shown to differ in no significant way from those obtained from secondary tumors arising on bacteria-inoculated

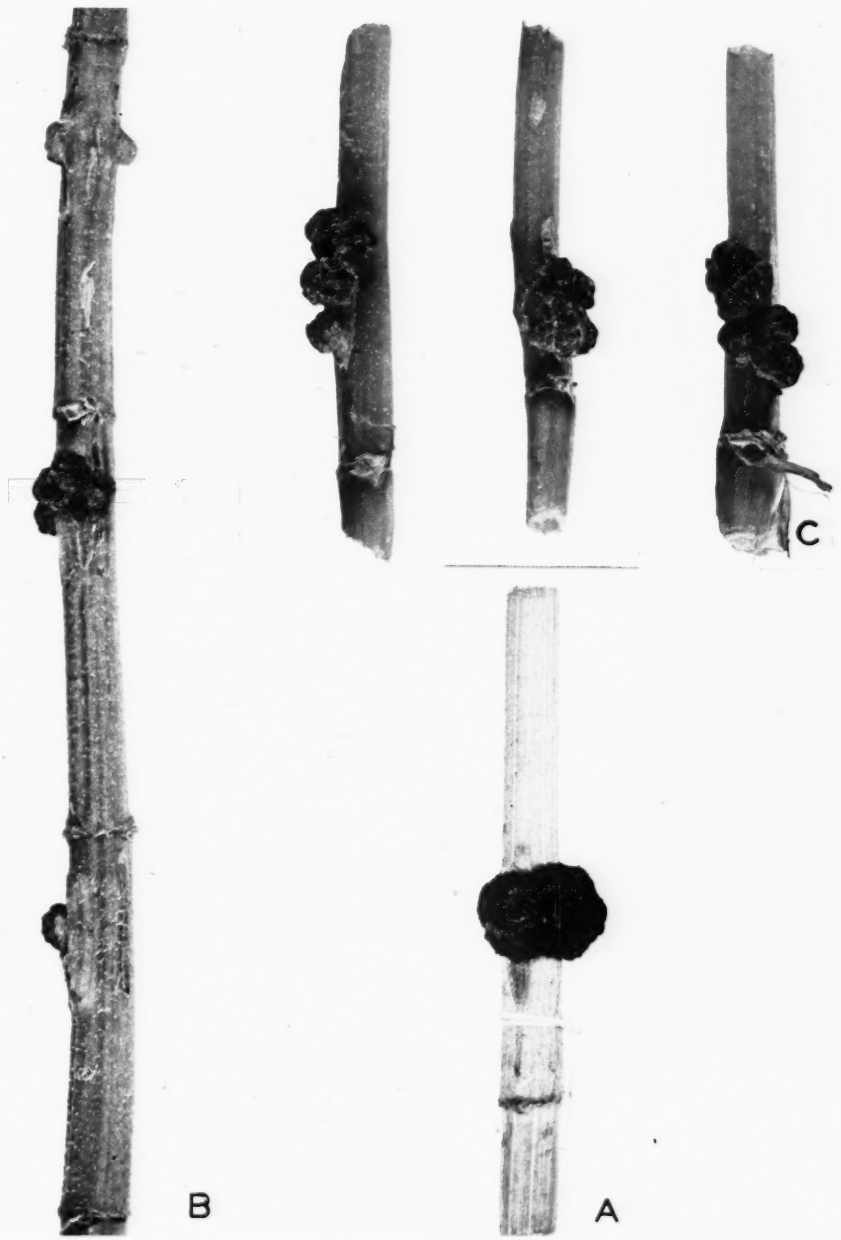

FIG. 8.—Examples of induced bacteria-free tumors. (A) Produced on sunflower by ingrafting sunflower tumor-tissue culture from a secondary gall. (B) A series of 4 tumors on artichoke produced by implanting sunflower tumor-tissue cultures at 3 sites (heteroplastic graft). (C) A series of 8 tumors produced on sunflower by ingrafting 9 fragments of tissue taken from artichoke tumors which were in turn produced by ingrafting sunflower tumor tissue cultures (heteroplastic graft). Passage through artichoke has not reduced the tumor-forming capacity of these tissues for sunflower. Mag. × 1.

plants. On July 15, 1941, a more extensive series of 100 cultures, all of a single clone and all in the 10th *in vitro* passage, was tested. Pieces of culture about a millimeter in diameter were used. Four methods of inserting the grafts were tested. Only a single tumor was produced from 25 grafts carried out by each of 2 methods. Four tumors resulted from 25 grafts by a 3rd method, while the 4th method produced 13 clean-cut tumors, 4 additional growths of clearly tumorous character, 4 abnormally healed wounds, and only 4 wounds showing no significant reaction. The result by this last method was thus 52 per cent of undoubted takes and an additional 32 per cent which are to be considered probably positive. Ten similarly executed grafts of tissue taken from the pith of healthy plants and serving as controls all healed normally and were recorded as 100 per cent negative.

Cultures of the series from which these grafts were made were retained and allowed to form considerable masses of tissue, and on Sept. 10, 1941, 30 fragments were grafted into 10 young plants of artichoke (*Helianthus tuberosus* L.) (heteroplastic grafts) using the double-incision method which had proved most satisfactory before. Of these, 19 (60 per cent) formed definite tumors. Pieces of tissue from 9 of these tumors were excised on Nov. 29, 1941, and grafted back into sunflower plants in 18 grafts. Ten (55 per cent) had formed tumors by Jan. 15, 1942.

to tumor formation. In view of the obvious difficulties in establishing vascular connection, this percentage is satisfactory. It is certainly too high to be ascribed to chance contaminations with crown-gall organisms. The character of the growth obtained is likewise quite different from that caused by contaminations, being closely localized in contrast to the diffuse tumefactions which result from contaminations of large wounds. The occurrence in individual experiments of 50 and 60 per cent of takes after as many as 25 passages *in vitro* furnishes unequivocal evidence that

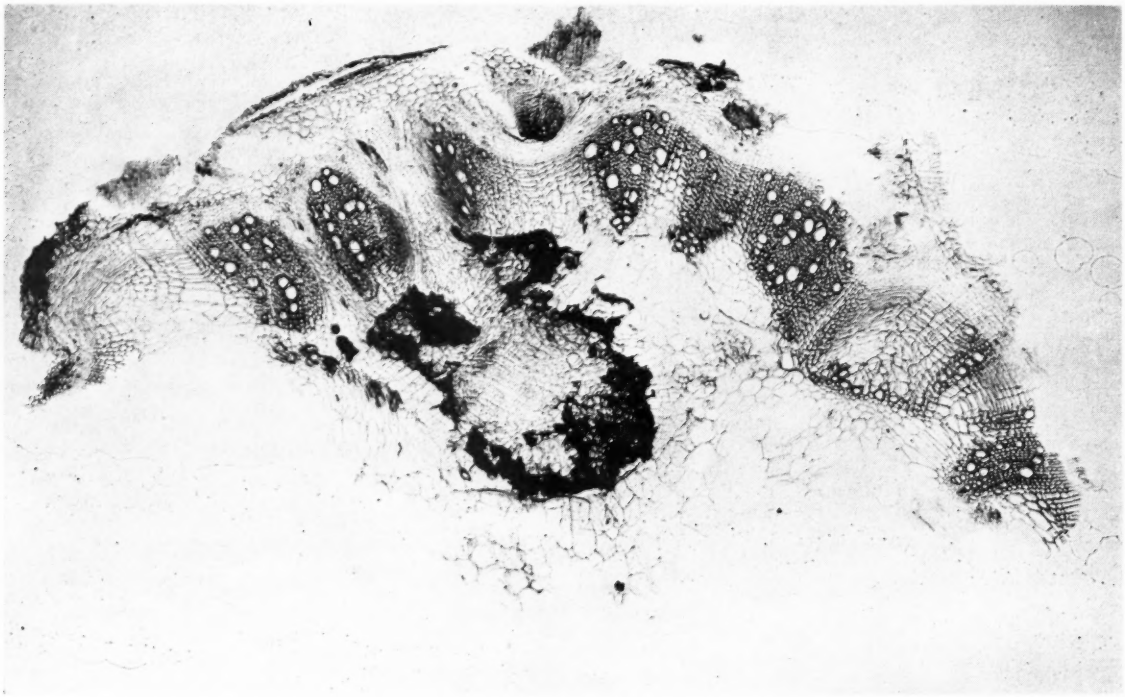

FIG. 9.—Transverse section of a sunflower stem 6 weeks after implantation of a fragment of tumor-tissue culture (passage 9). The fragment was implanted in the outer portion of the pith, too deep to make an early and satisfactory vascular connection with the host. While some growth occurred at the outer margin of the implant, no tumor was formed, the implant is already partly necrotic, and the original wound has completely healed. Freehand section. Mag. × 26.

A 2nd series of 100 cultures (15th passage) was grafted on Sept. 23, 1941, into sunflower plants. The plants, because of the reduced sunlight at this season, were not in ideal condition. At the end of one month (Oct. 21), 19 had formed good tumors. The plants were in too poor condition to be retained further. In a 3rd series of 100 (passage 20), set up on Dec. 2, only 2 implants formed tumors, while a 4th series of 100 (passage 25) carried out in the good growing weather of March and April gave 31 unmistakable takes.

Similar grafts with isolations H_{13}, 10, 20, 22A, and 22B all produced typical tumors. Of a total of 601 grafts made to date, 133 formed definite and unmistakable tumors and a considerable number more healed in such a manner as to suggest a tendency

these tissues, demonstrably free of bacteria and showing a definitely abnormal proliferative power both in the original host and *in vitro,* are likewise capable of producing characteristic crown-gall tumors when grafted into either sunflower (*Helianthus annuus*) or artichoke (*Helianthus tuberosus*). The tumors may be passed back and forth between these 2 hosts at will. Control experiments were carried out in which similar grafts were made using pith fragments and procambial tissues of sunflower, tissue cultures of healthy sunflower cambium, tissue cultures of cambium from indole-oxalo-acetic acid tumors on sunflower, and tissue cultures of the genetically induced tumefactions of hybrid *Nicotiana langsdorffii* Weinm. × *Nicotiana glauca* Grah. plants. All healed without forming tumors. Tumors were produced only in the

presence of tissue derived from tumors previously induced by bacteria, whether or not these tissues were themselves bacteria-free when used.

The artificially produced, bacteria-free growths, as can be seen from the accompanying figures, were typical crown-gall tumors, both in gross appearance and in section. The surface was frequently, but by no means always, somewhat smoother than in bacterial tumors. This suggests that the proliferative impetus, since it has presumably become a characteristic of all tumor cells, enjoys a more uniform distribution than is true in primary tumors where this impetus comes from varying concentrations of bacteria to be found in scattered pockets distributed through the affected tissues. This characteristic uniformity was carried over into the internal structure. The great mass of the tissue was made up of thin-walled parenchymatous cells which were mostly isodiametric but arranged in rows radiating outward from the location of the original implant. Scattered among these were thicker-walled, modified scalariform cells which occurred either in isolated masses or in short, radially oriented strands. Typical wood was not formed except in the "collar" region which appears to arise not from the implant but from the host xylem. In old tumors the scattered vascular elements seem to be concentrated in concentric bands, giving the appearance of a Liesegang effect. The outer 2 or 3 mm. of the tumor was commonly a definite cortex of rapidly dividing cells without scalariform elements.

The fusion between tumor and host was for the most part very intimate so that, except for occasional necrotic cells at the line of the original cut, the two could not be sharply differeniated except by the relatively smaller, more irregular cells and slightly larger nuclei and the absence of typical wood in the tumors. Chromosome counts showed that the tumor cells were not, as seemed possible, polyploid. The chromosome counts were regularly normal. Binucleate cells occurred quite commonly, as well as occasional cells with many nuclei, and nuclear outlines were sometimes distorted, suggesting a tendency to abnormal behavior, but these aberrations were too rare to represent any certain characteristic of the tissues. Multipolar spindles have not been observed. At the graft union small groups of tumor cells sometimes pushed out into the host, either into regions where the host cells were large or by crushing the host tissues, but these intrusive growths, as is to be expected in a plant, seldom penetrated to depths of more than a few hundred microns and probably do not differ significantly from those observed in normal graft unions. *There is no evidence that the host cells were themselves modified in their behavior by the presence of adjacent tumor cells in any manner other than would be the case in the presence of normal cells and under the mechanical stresses resulting from adjacent abnormally active growth. The tumors appear to arise largely, if not entirely, from pre-existent tumor cells.* This represents additional evidence that neither *Phytomonas tumefaciens* nor a tumefacient virus was here involved.

DISCUSSION

Sunflower plants, upon inoculation with a virulent strain of *Phytomonas tumefaciens,* first produce rapidly growing, massive galls localized immediately around the point of inoculation. These contain in their tissues considerable numbers of bacteria of the type used as inoculum. The galls are the direct result of the presence of these bacteria, though by what specific means, whether chemical or mechanical, remains to be discovered. This reaction sets in immediately following inoculation, visible proliferation occurs within a few days, and the resulting growths are frequently the only outcome.

In a considerable percentage of plants so inoculated —from 20 to 75 per cent, according to the condition of the host and doubtless to many unknown factors as well—there follows, however, a much slower reaction which we believe to be of great significance. There first appear at irregular intervals in the first internode above the point of inoculation swollen areas subjacent to the vascular bundles, which with their connecting tissues are known as "tumor strands." Bacteria can sometimes be isolated from them but are usually not demonstrated by the ordinary methods. They must obviously be present in small numbers, if at all, and there is reason to suspect that they may often be entirely absent. There is here more than a suggestion that these strands may result, not from immediate contact with bacteria themselves, but from diffusible products from the bacteria which exert their influence at a distance from the point of origin.

Subsequently, there frequently appear in the petioles, midribs, large veins, and at the point at which the leaves are attached to the stem, 1, 2, 3, and sometimes even 4 nodes above the point of inoculation, localized swellings which are in the great majority of cases entirely free of bacteria. These are the so called "secondary tumors," which partake at least superficially of the nature of the metastases in animal tumors. Their origin, although causally related to the presence of bacteria in the host, is apparently only secondarily so related, the bacteria themselves rarely, if ever, occurring at the points of reaction.

We have demonstrated by several methods that these metastatic tumors are for the most part bacteria-free. In the first place, tissues therefrom, when excised under aseptic precautions, ground, incubated in broths

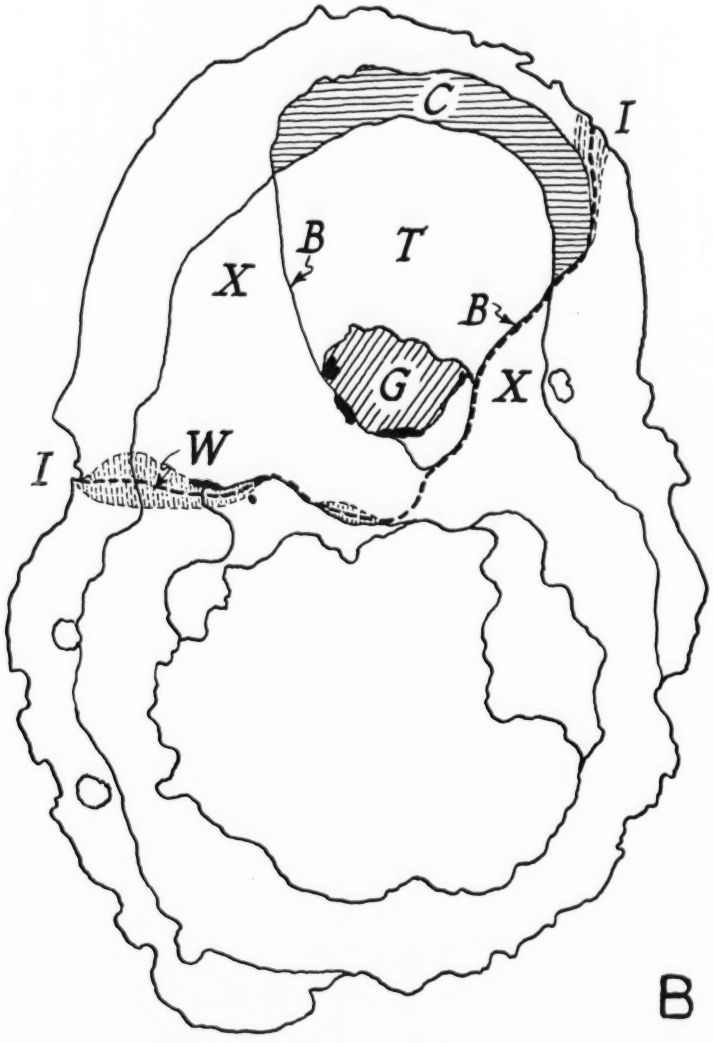

FIG. 10.—(A and B) Transverse section of a sunflower stem carrying a well developed but unerupted tumor. The position of the original incision is betrayed by the breaks in the continuity of the epidermis at the 2 points marked *I*. The wound has healed throughout its length, at least at this level, and is only recognizable by a narrow band of wound tissue at *W* and some other isolated points and by occasional necrotic bands. The original implant *G* was evidently placed (as the reader faces) slightly to the right of the plane median to the 2 vertical incisions and in excellent contact with the vascular ring. Its inner face is still recognizable by a line of necrotic cells, but the outer margin has grown extensively, pushing outward and forming the tumor *T*. The boundary *B* between tumor and host is quite sharp. The developing tumor has crushed the host cortex at *C* and, if left to continue development, would have eventually erupted at this region. The mechanical strain which the presence of an abnormal mass of tumor cells has placed on the neighboring host tissues has resulted in extensive hypertrophy of the xylem at *X*, forming a collar around the tumor but histologically quite distinct from the latter. This hypertrophied host xylem is often recognizable in even very old tumors. The contrast between the manner of tissue development in the presence of the implant at *G* with the behavior in the absence of such an implant at *W* is worth emphasizing. Mag. × 18.

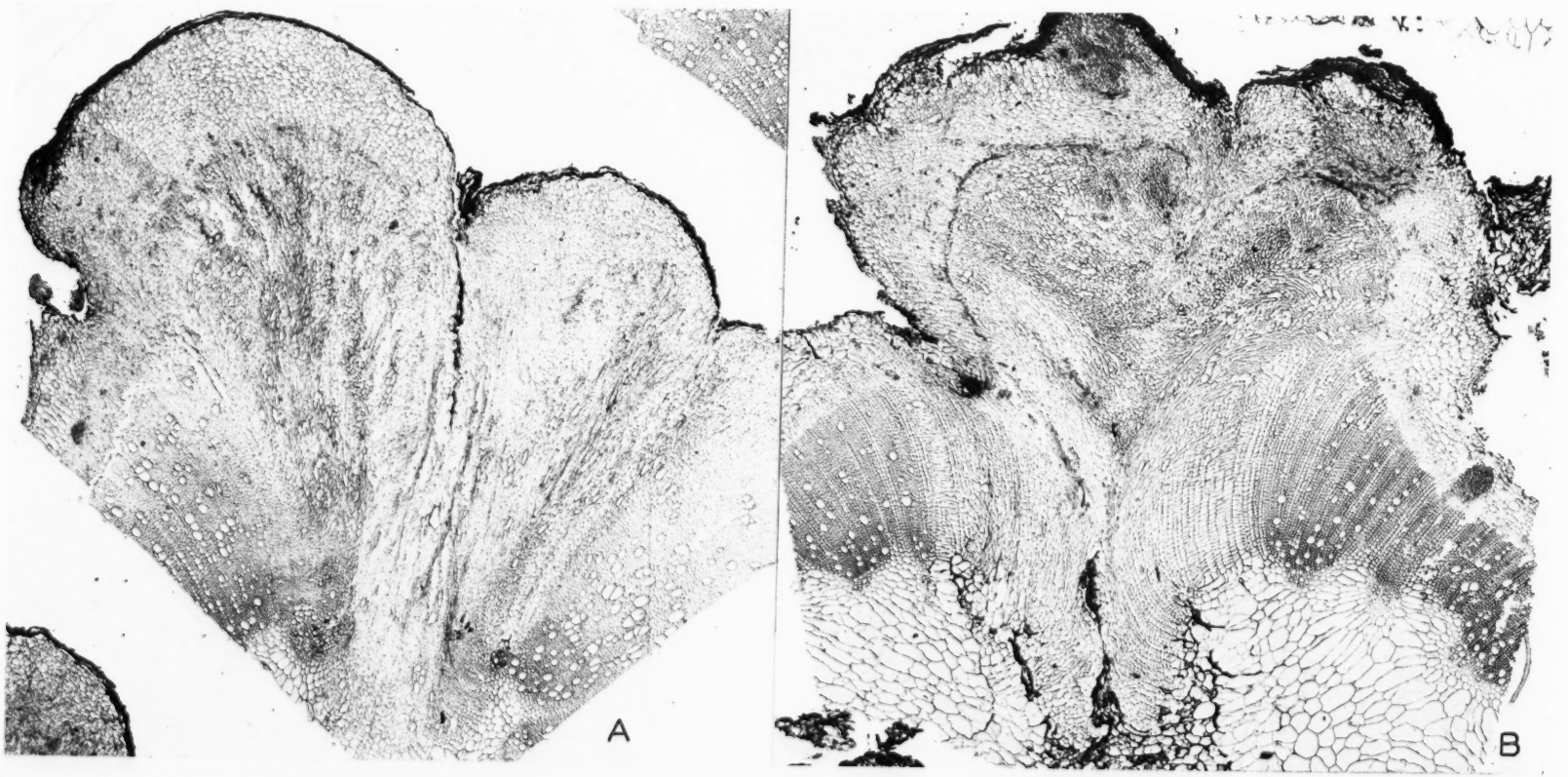

FIG. 11.—(A) Transverse section through a young tumor produced by needle puncture inoculation with crown-gall bacteria. The line at which the needle penetrated is marked by necrotic cells at the center of the tumefaction. The tumor proper is made up of disoriented scalariform cells in a great mass of parenchyma. It is bounded at the outer face by a characteristic parenchymatous cortex and is flanked by hypertrophied host xylem indicative of the mechanical strains set up by the presence of an abnormal mass of cells. (B) A bacteria-free tumor resulting from implantation of a tumor tissue culture at the lower center. This tumor shows the same disoriented, mixed scalariform and parenchyma stroma, the parenchymatous cortex, and the flanking hypertrophied host xylem as that shown in the bacterial tumor. Mag. × 18.

of various kinds, and then cultured in a variety of liquid and semisolid nutrient media known to support growth of *Phytomonas tumefaciens,* have not yielded cultures of this organism except in such small numbers as one obtains from apparently healthy tissues taken from other parts of infected plants. The rare colonies of bacteria appear to be contaminations probably not causally connected with the occurrence of tumors. In the second place, fragments of secondary gall tissue, when thoroughly ground aseptically and then inoculated into young sunflower or tomato plants, seldom produce galls, while similarly treated primary gall tissues containing crown-gall bacteria regularly produce rapidly growing invasive galls.

Tissues from secondary galls have been isolated as tissue cultures and caused to multiply *in vitro* to an extent of many thousands of times through more than 30 passages in constant contact with a nutrient known to support a profuse growth of *Phytomonas tumefaciens,* without ever producing colonies of this organism. These cultures have themselves been subjected to bacteriological tests such as are regularly used for isolating the organism from other host tissues, with consistently negative results. Furthermore, the cultures have been tested serologically, by first using them as antigens in building up an immunity in rabbits to sunflower tissue and to any organisms contained therein, and then testing with cultures *Phytomonas tumefaciens* of the same strain as that used in the production of the primary galls, with consistently negative results. The universally negative results of more than 2,000 tests of various sorts cannot but be significant.

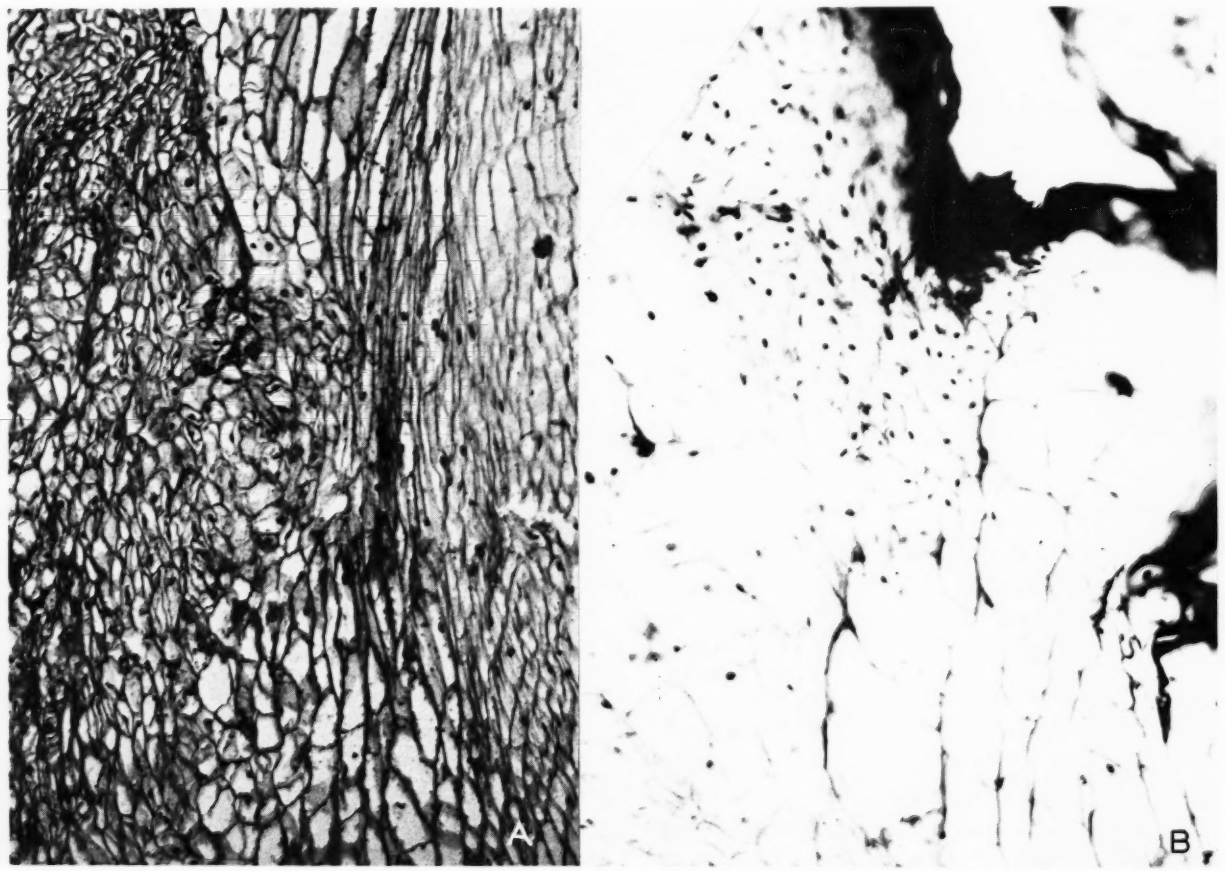

FIG. 12.—(A) Host-tumor boundary between cells formed from a sunflower tumor-tissue culture (left) and a sunflower host (right). The 2 merge completely in the lower half of this picture, although at the center is a disoriented group of cells which seem to have invaded and crushed the host cells instead of merging with them. Mag. × 120. (B) A similar host-tumor boundary but between cells formed from a sunflower tumor-tissue culture (left) and an artichoke host (right) (heteroplastic graft). The boundary between small sunflower tumor cells at the left and the larger artichoke cells at the right is quite sharp. No invasion or crushing is evident. Mag. × 110.

The occurrence of galls in itself represents a deviation from the normal behavior of sunflower tissue, a tendency to escape from the morphogenetic restraints normally applied to these regions. In the absence of any causative bacteria, we must assume that this abnormal behavior originally results from the presence of some stimulating material having its primary origin

in the bacterial colonies or in the primary gall tissues subjacent to these colonies.

The occurrence of gall masses dependent on obvious stimulation would not, in itself, seem so extraordinary. Galls resulting from the continued action of both chemical and physical irritants are well known. In the study of the galls of the type under consideration here, however, there has come to light a characteristic which is, to our knowledge, not shared by any other known plant material and which seems to us comparable only to characteristics of animal materials classified as cancers. This is their complete freedom from normal morphogenetic restraint, their capacity for unlimited, disorganized growth not only in the original host but in other hosts and *in vitro* as well, *in spite of the absence of any recognizable invader.* This behavior can hardly be the result of residual stimulating material carried from culture to culture in the process of transfer. A dilution factor of 10^{-14}, although short of the theoretical molecular limit of 10^{-23}, is still so close to that limit that the undiminished proliferative impetus is unlikely to be caused by this small theoretical residuum. The abnormal proliferative capacity and the capacity to produce tumors have become part and parcel of the nature of the cells themselves, quite independent of the original inciting agent, the bacteria or their immediate products. These are qualities which, when they appear in animal cells, are used to distinguish cancerous growths from healthy or merely inflammatory tissues.

In drawing analogies between these growths and animal cancers one must, of course, take care that the comparisons are restricted to levels at which they are possible within the structural limitations of the two classes of organisms. The authors lay no claim to authority in the field of oncology and must speak here in the capacity of laymen. Many readers of this paper may, on the other hand, be unfamiliar with certain features of plant morphology. It seems desirable, therefore, to present some discussion of pertinent terms and of the properties of the organisms involved. Webster's Unabridged Dictionary defines "cancer," in the sense in which it is universally used by laymen, as "Any malignant tumor." It defines "tumor" as "An abnormal mass of tissue, not inflammatory and independent in character, arising without obvious cause from cells of pre-existent tissue, possessing no physiologic function and characteristically unrestrained in growth and structure." "Malignant" is defined as "Tending or threatening to produce death." If one combines these 3 definitions, one obtains a characterization the essential feature of which is the capacity for indefinite, disorganized growth independent of any restraining influence of the host organism. Invasiveness, malignancy, and capacity for metastasis all arise from this fundamental irresponsibility.

This characterization is of such a general nature that it can be expressed in plant tissues as well as in those of animals. But the expression in plants will inevitably differ in many ways from the corresponding expression in animals as a result of the differences in developmental and structural characteristics of organisms belonging to the 2 categories. (We are, of course, tacitly restricting ourselves here to the "higher" plants and animals.) It is, therefore, necessary to outline the physical bases on which this expression must rest and the form which abnormal growth may be expected to take, and to call attention to those expressions which cannot occur within the limitations of plant structure.

First.—The animal cell is characteristically a mobile organism, surrounded by a tough pellicle capable of considerable extension and unlimited distortion without rupturing and which may, by processes arising largely internally, be pierced and reconstituted repeatedly, as exemplified in phagocytosis and in the ejection of excretory vacuoles, without destroying the capacity of the cell to function. Adjacent cells are not fixed rigidly to one another and are constantly gliding over one another. The cells pass through a fairly rapid cycle of youth, maturity, senescence, and death, and are constantly being replaced so that, with the exception of nerve cells, no functioning animal cell is ever more than a few months old, no matter in how aged an individual it is to be found.

This mobility provides a means by which animal tumors may penetrate deeply into adjacent structures, the tumor cells creeping actively between healthy cells of the host. As a result, a developing carcinoma may extend irregularly to considerable depths into the subjacent organs, particularly along such channels as the lymph nodes, providing one method by which the so called permeation metastases occur. The cell's mobility also makes it highly resistant to mechanical injury. An animal tissue can be ground "thoroughly," squeezed through coarse-meshed screens, and even passed through moderately coarse filters, and still may contain considerable numbers of viable cells. Consequently, in the study of tumor transmission particular precautions must be taken to distinguish between the transmission of infective agents and of viable tumor cells.

The constant renewal of cells within the organism carries as a consequence that, although carcinoma is a disease of late middle life, it is *not* a disease of old cells, and one must look to the characteristics of the *extracellular,* though intraorganismal, features of postmature individuals rather than to any characteristics of the cells themselves for an explanation of their predilection for carcinoma.

The characteristics of the plant cell are in decided contrast to all of these features. Each cell is surrounded by a cellulose wall which in most cases becomes lignified, suberized, or otherwise thickened and modified at a very early stage. This wall is capable of only gradual extension and very restricted distortion and is consequently relatively easily ruptured. The protoplast, which is normally closely appressed to the wall, has no appreciable pellicle and is easily injured once the protecting cellulose wall is broken. The cells are rigidly cemented one to another into a continuous skeletal framework and, except in certain specialized coenocytic tissues such as immature endosperm, plant cells never move freely one upon another.

Moreover, plant cells are never replaced during the life of the organism, except under pathological conditions. Once formed, a cell persists in its original location until death, and its skeletal framework often persists thereafter. Individual pith cells in the giant cactus have, for example, been shown to remain undivided and to function normally after more than a century. From these characteristics it is clear that movements of the sort involved in the spreading of a tumor must, in a plant, take place not by active cell migration but by transformation of pre-existent cells (infection), by slow digestion such as occurs in the presence of parasites or in the progress of a haustorial cotyledon into the endosperm, or by mechanical crushing and rupturing of the subjacent tissues. It is to be anticipated that penetration by *tumor cells* will seldom be either deep or extensive. One would anticipate as a result of these facts that a plant tumor, except where derived from a motile invading organism, would have much more definite, clean-cut outlines than an animal tumor.

Plant cells, as a result of the rigidity of the walls and the lack of a tough protoplasmic pellicle, are highly susceptible to mechanical injury, so that moderate grinding can be relied on to destroy most if not all viable cells.

Moreover, since plant cells are not renewed, any infection of old regions of a plant, such as the pith, is an infection of *old, highly differentiated cells*. The pith and other old parts, for this reason, are relatively resistant to many types of infection. Tumors can usually be induced most easily in rapidly dividing regions such as growing points, cambiums, etc. This does *not* mean that the susceptible plant protoplast differs notably in its characteristics from the susceptible animal protoplast. The level at which this particular difference occurs is an *extracellular* level.

Second.—One of the characteristic features of all higher animals is their division into well defined tissues and the relative stability of these tissues. Epithelium, endothelium, connective tissue, bone, nerve, blood are all sharply distinct cellular entities and retain their identities even under highly abnormal external conditions. The stability of strains of fibroblasts from different organs of a single organism is pronounced. And, since animal tumors ordinarily affect particular tissues, it is possible to classify them as epitheliomas, myomas, osteomas, leukemias, etc., and to identify them on a histological basis.

Here again the plant presents a decided contrast, for, while we recognize parenchyma, cambium, xylem, endosperm, etc., as plant *tissues,* these do not represent aggregates of cells of a single stable morphological type and a single function. A plant cell may during its lifetime be transformed from one morphological type and function to another. In fact, Haberlandt pointed out many cases in which long cells belonged histologically and functionally to one "tissue" classification at one end and to quite another classification at the other end. It is, therefore, futile to look for spindle cell or round cell tumors, fibromas, epitheliomas, etc., in plants. The anatomical basis in which neoplasia must be expressed precludes the possibility of establishing such categories.

Third.—The circulatory system and the relation of endothelial cells to this system in animals constitute a characteristic feature of their organization which is of great importance in the development of tumorous growths. This system ramifies to all regions and into all tissues of the organism. The fluid within it is in constant, rapid, circulatory motion and the cells of which the walls of these channels are made are living active cells capable of considerable mobility. These facts, together with the capacity of animal cells to move about, to push between one another, and on occasion to penetrate to the lumina of veins, arteries, lymph channels, etc., make possible the movement of "cancer" cells from a primary focus into all parts of the body, resulting in the phenomenon of transport metastasis.

Here again the plant differs significantly. There is no *circulatory* system. There is, it is true, a system of vessels which serves to carry water and dissolved materials, generally in an upward direction (xylem). And there is a second system of cells which serves to carry elaborated foods, generally in a downward direction (phloem). But these systems are not connected directly to one another. The phloem, while made up of living cells, is so constituted that the open channel is continuous only within each individual cell and is broken at the end of each cell by a sieve plate and a presumably continuous protoplasmic barrier through which a tumor cell, even if it could conceivably penetrate to the interior, could not possibly pass intact to another unit in the phloem

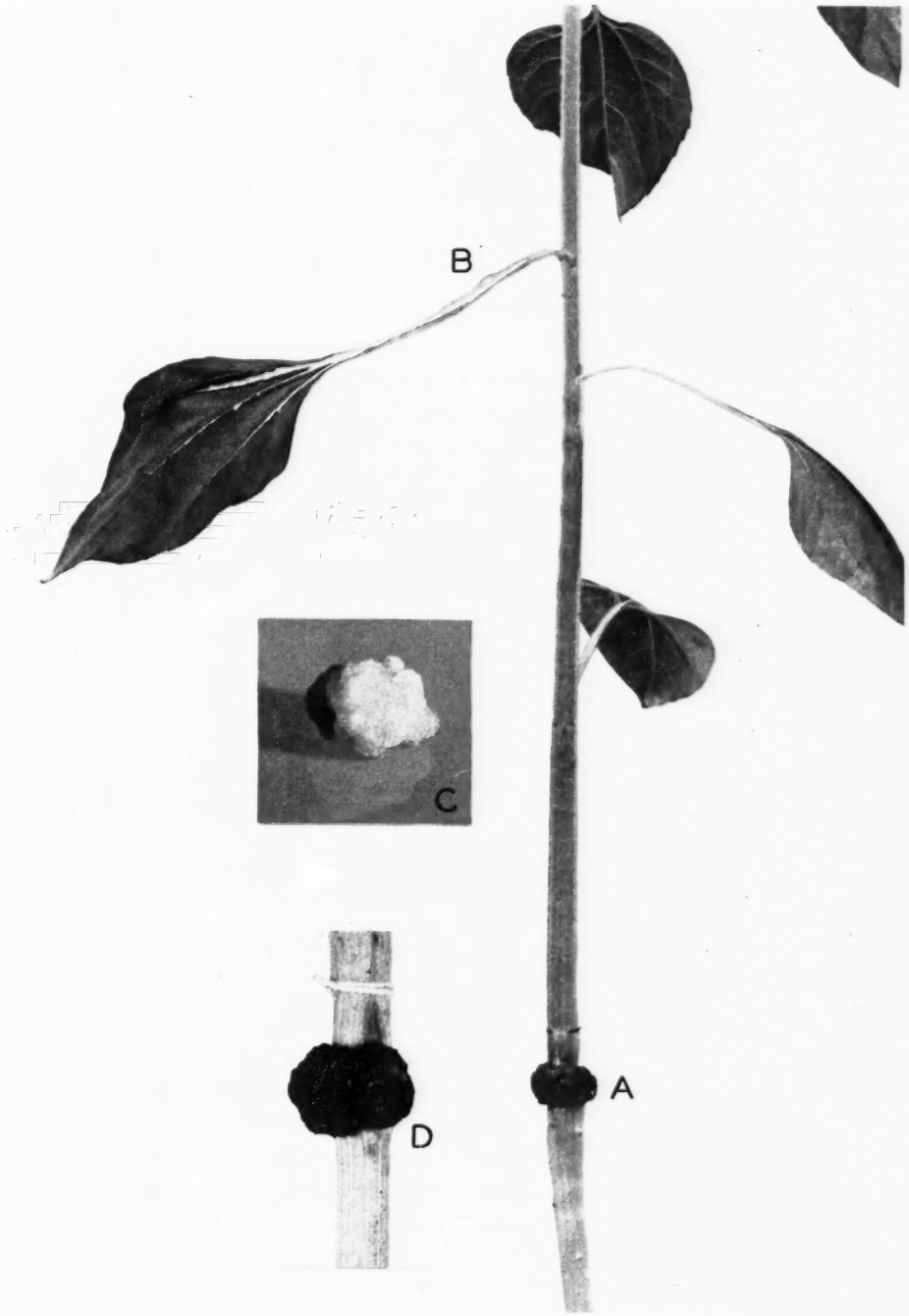

FIG. 13.—Recapitulation. The primary tumor at *A* was produced by inoculation with crown-gall bacteria. Some stimulus of a nature as yet unknown passed from this focus up through the stem, giving rise to tumor strands, unerupted stem tumors, and finally secondary tumefactions on the petiole and large veins and at the base of the petiole *B* of the 4th leaf above the primary tumor. The lowest of these 4 leaves had fallen leaving only a scar. Bacteria-free tissue cultures *C* of rapid and disorganized growth were isolated from such secondary tumors and cultivated *in vitro* through many passages. When fragments of such tissue cultures were implanted under the bark of young healthy sunflower plants, they produced new tumors *D* of a type indistinguishable from the primary tumors except that they were completely free of the original tumefacient organisms.

"channel" unless it were very small indeed. The xylem is more nearly continuous, but the open cells are dead and the channels are lined with thickened inert cellulose or lignified walls through which tumor cells could either enter or escape only by slow growth processes. It is, therefore, improbable that metastases resulting from transport of tumor cells would occur in plants.

Fourth.—Malignancy, in the animal organism, is a direct result of the definite invasive character of animal cancers. These growths, although they may start in relatively unimportant regions or tissue layers, tend to push out into, disrupt, and finally replace vital organs. The animal has only one liver, one pituitary, one central gut. When this is attacked and destroyed, its loss is fatal to the entire animal. The plant, on the other hand, does not possess *single* organs but a multiplicity of similar regions and organs—conducting strands, leaves, growing regions, etc. The possibility that the attack on any particular region or organ by a tumor may be fatal to the organism as a whole is therefore restricted. Certain plants such as the beet do, it is true, have very small, concentrated terminal growing points from which all growth of leafy organs necessary for the survival of the plant must come. If a tumor forms in such a region it is generally fatal, even though it may be relatively small. In this respect it is comparable to the lethal tumors of animals. Tumors on the stems of plants, and especially on the crowns and trunks of trees, may invade such a large proportion of the cross section as to stifle and destroy the host. It is, nevertheless, evident from what has been said that at the level of *the organism as a whole* "malignancy" is essentially dependent on host topography and that the topography of plants, while it does not preclude the existence of malignant tumors, does make them relatively less frequent than in animals. And this in spite of the fact that the affected *cells* may possess exactly comparable qualities in the 2 groups of organisms.

There are, thus, obviously a number of features commonly used in the differentiation of animal tumors and cancers which rest upon developmental or functional characteristics that are more or less restricted to animals and cannot be carried over and applied to plant tumors. Plant tumors cannot be sarcomas, carcinomas, epitheliomas, etc. Plant tumors cannot be expected to spread extensively as permeation metastases, nor are they likely to spread at all as transport metastases except when transport is brought about artificially by the experimenter. The characteristics of invasiveness and of malignancy must be interpreted loosely in any definition which is to apply to members of both kingdoms. But the essential characteristic, of *being able to grow independently of any morphogenetic restraint,* upon which all other diagnostic features ultimately depend, since it is a characteristic of the cell itself, is equally capable of expression in neoplasia of all higher organisms. It is with these facts clearly in mind that the significance of the results presented in this paper must be judged.

The growths which have been studied in this work arise from cells of the host under the influence, expressed in some manner as yet unexplained, of the presence of bacteria elsewhere in the plant. Once established, they become completely independent and capable of rapid growth in artificial media as well as in homologous and heterologous grafts. Their growth and structure become unrestrained, both quantitatively and qualitatively, so that they produce typical tumors. These tumors and their tissues, whether *in vivo* or *in vitro,* are unphysiologic and potentially injurious as well as free from morphogenetic restraint. These are all qualities which are commonly attributed to cancer cells. The invasiveness of these growths is restricted by the structural characteristics of the host but is expressed to as great a degree as is to be expected within these structural limitations.

Metastatic and transplanted tumors have not, in the brief periods during which they have been under observation, "produced death." Nevertheless we believe that they do possess the properties which, in animal cancers, *"tend* to produce death." Those properties, as pointed out above, are, first, invasiveness, the tendency to penetrate, disrupt, and finally displace vital organs; and, second, the capacity to move from primary foci at harmless points to scattered, secondary foci in and adjacent to vital organs from which invasion can "produce death." The invasiveness is, in plant cells, limited structurally. The capacity to move to new foci is likewise structurally limited. Nevertheless, in carrying out transplantations or grafts we have artificially overcome those structural limitations and have produced what are essentially artificial transport metastases. The fact that such grafts "take" with high frequency, producing massive tumorous growths, is, we believe, a demonstration that the essential qualities for metastasis, as far as the *cell* is concerned, do exist in these cells, capable of clear expression under experimental conditions but limited in nature by host characteristics. Not all animal cancers metastasize. Nor do all cancers which metastasize in the original host do so when transplanted to another animal. Their malignancy is limited by host characteristics and is evident only under special conditions. Such appears to be the case with these plant cells.

We possess here a plant material which shows all the essential characteristics of a cancerous neoplasm. This material can be produced at will in unlimited

quantity, and there is reason to expect that the processes by which these cancerous qualities arise may be followed experimentally. We believe that we have here something which may well render great service in the study of the origin and character of those changes in cell behavior which are considered to be the basis of "the cancer problem."

SUMMARY

Secondary or metastatic tumors frequently arise on sunflower plants inoculated with *Phytomonas tumefaciens* at considerable distances from the primary tumefactions. These have been shown by cultural and serological methods to be characteristically bacteria-free. Tissue cultures isolated from these tumors show a rapid disorganized type of growth contrasting sharply with the slow, moderately organized growth of those isolated from healthy tissues. Upon implantation into uninfected plants of the same or related species, these tissues produce typical crown-gall tumors. This capacity for unrestrained, invasive, potentially malignant growth both *in vivo* and *in vitro* in the absence of the original excitant differentiates these tissues from any other plant materials reported to date and places them in a category comparable to cancerous growths in animals.

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Human Neoplasms in Tissue Culture*

Dale Rex Coman, M.D.

(From the Department of Pathology, University of Pennsylvania Medical School, Philadelphia, Pa.)

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The tissue culture method has been successfully applied to the study of human neoplasms only in recent times. The cultivation of human neoplastic tissue *in vitro* was first attempted by Carrel and Burrows (2) in 1911 without much success. Maccabruni (8) in 1913 observed proliferating cells around human fetal uterine and ovarian tissues planted in plasma. In 1914, Losee and Ebeling (7) kept human sarcomatous cells alive and growing in human plasma for more than 2 months.

Since these earlier attempts much progress has been made, and it has become apparent that the cultivation of human neoplasms offers no obstacles not also encountered in the cultivation of neoplasms of lower animals. The origination of the roller tube method of tissue culture by Gey (4) and its development by Lewis (6) have made possible the maintenance of more massive cultures over extended periods.

There are certain advantages in a method whereby living neoplastic cells can be directly observed. For instance, opportunity is afforded for the direct observation of cellular movements. The formation of patterns of growth can be studied. By the roller tube method especially, chemical investigations can be carried out upon the fluid medium in which the cells have been living. The effect of substances which may stimulate or inhibit growth of the tumor cells can be observed. The chemical and physical requirements for the continued growth of human neoplastic cells may be investigated. The process of differentiation and organization of cells might well be studied by tissue culture.

The present paper deals with the growth patterns observed in a wide variety of human neoplasms maintained *in vitro*, and serves as the introduction to a series of studies now under way on human tumors.

* This investigation was aided by grants from the Faculty Research Committee of the University of Pennsylvania and from The International Cancer Research Foundation.

MATERIALS AND METHODS

The majority of tumors cultured were obtained from the operating room of the Hospital of the University of Pennsylvania, through the courtesy of Dr. I. S. Ravdin and his staff.

Tissues were collected in sterile dishes and planted as soon as possible. It was found, however, that tissues could be stored overnight in the refrigerator without noticeably affecting their subsequent proliferation in culture. A few of the tumors were obtained at autopsy. Large, solid blocks of tissue were placed in 95 per cent alcohol for a few minutes and then fragments from the deeper parts were removed aseptically for culture. Successful cultures resulted from tissues obtained as long as 27 hours after death.

All tissues were cut into small pieces, up to 5 mm. in diameter, and washed in Gey's solution (5) before planting. Two or 3 drops of heparinized chicken plasma were allowed to run down an ordinary pyrex test tube, forming a streak along the inside of the tube. The tissue fragments were placed in the mouth of the tube with small forceps and then arranged in a row in the chicken plasma with a long pipette having a slightly curved tip. The plasma was clotted by adding a drop of chick embryo extract. A fluid medium was added, consisting of 8 drops of human fetal serum and 5 drops of Gey's solution. The tube was closed with a rubber stopper and placed in a rotating device (3) housed in an incubator at 37° C. This rotator turns about 9 times per hour, causing the fluid medium to wash over the embedded fragments of tissue, thus furnishing a primitive type of circulation. The fluid medium was withdrawn every 4th day, or more frequently if necessary, and replaced

DESCRIPTION OF FIGURES 1 TO 6

FIG. 1.—Hematoxylin and eosin stained section of an adenoma of the breast for comparison with the other photographs in the series which show the same tumor *in vitro*. Mag. × 440.

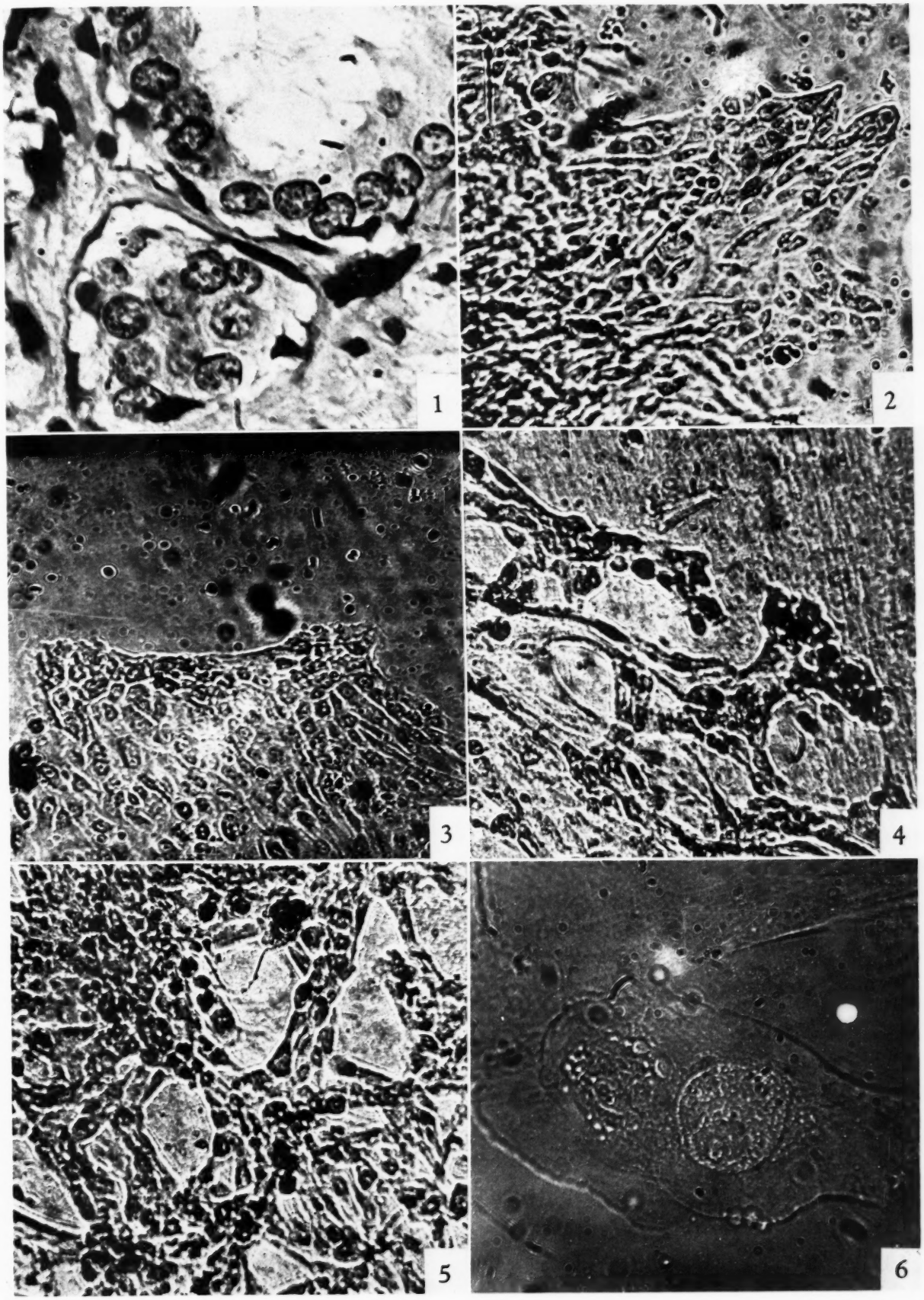
FIG. 2.—Epithelial cells *in vitro* forming a tongue-like process, characteristic of the early stages of culture. Mag. × 113.

FIG. 3.—Epithelial cells forming a broad sheet. Mag. × 113.

FIG. 4.—Epithelial cells showing beginning acinar arrangement, after a month of culture. Mag. × 113.

FIG. 5.—Epithelial cells showing acinar pattern at a somewhat later stage than seen in Fig. 4. Mag. × 113.

FIG. 6.—An isolated epithelial giant cell *in vitro*. Such cells are capable of ameboid movement. Mag. × 550.



FIGS. 1-6

by fresh medium. Liquefied areas in the plasma were patched with fresh plasma and chick embryo extract. Subcultures to other tubes or to hanging drop cultures were made as desired, or when thought advisable for the health of the culture. The outgrowing cells were observed and photographed directly through the walls of the tubes. Of each tumor, part was placed in formalin, and hematoxylin and eosin stained preparations were made for comparison with the growth pattern developed *in vitro*. The period of cultivation varied from 1 to 7 months, and many cultures were actively proliferating with no apparent decrease in vigor when discarded.

RESULTS

In Table I are listed the human neoplasms maintained *in vitro* by the method described above. A discussion of the growth patterns and cell types produced by these tumors follows:

TABLE I: HUMAN NEOPLASMS GROWN IN TISSUE CULTURE

EPITHELIUM	
Benign	
Fibroadenoma, breast	4 cases
Cystic mastitis (involutional hyperplasia)	5 cases
Adenoma, pancreatic islets	1 case
Malignant	
Squamous cell carcinoma (branchiogenic)	1 case
Carcinoma, breast (primary)	9 cases
Carcinoma, breast (metastatic to lymph node)	3 cases
Carcinoma, stomach (metastatic to lymph node)	4 cases
Carcinoma, stomach (metastatic to liver)	2 cases
Carcinoma, prostate (metastatic to lung)	1 case
Carcinoma, adrenal (metastatic to lymph node)	1 case
Carcinoma, liver (primary)	1 case
SMOOTH MUSCLE AND FIBROUS TISSUE	
Leiomyoma, uterus	3 cases
LYMPHOID TISSUE	
Lymphosarcoma	4 cases
ENDOTHELIUM	
Angiosarcoma	1 case
PIGMENT CELLS	
Melanosarcoma	2 cases
MIXED TUMOR	
Parotid	2 cases

BENIGN EPITHELIAL TUMORS (FIG. 1)

Epithelial growth was never noted in cultures less than 72 hours' old and at times would start feebly, but usually became more vigorous after the first 2 weeks. The epithelial cells first formed tongue-like projections which later developed into broad sheets (Figs. 2, 3). These membranous sheets usually extended out over the surface of the plasma clot. Liquefaction of the plasma by the epithelial cells occurred particularly during the first few weeks of culture. Thereafter the cells showed little or no liquefying action even if kept without transplantation for several months. Acinar structures (Figs. 4, 5) were formed by the epithelial cells only after a month or so of culture. This acinar arrangement occurred whether or not fibroblasts were present and, as noted by Cameron and Chambers (1), the acini at times seemed actually to form better in the absence of the fibrous tissue. Occasionally, isolated giant cells were produced (Fig. 6). The final growth pattern assumed by the epithelial cells roughly approximated that of the original tumor as seen in the hematoxylin and eosin preparations.

In addition to epithelial cells, the benign tumors *in vitro* produced macrophages and fibroblasts. The macrophages emigrated during the first 24 to 48 hours, their number varying with different tumors, but always considerable. The number of these cells in all tumors is rarely appreciated in the usual histologic section. One is impressed by their consistent presence when tumors are examined *in vitro*.

Fibroblasts usually became evident by the 3rd day. These cells grew out in radial arrangement around the explants and in old cultures piled up in layers many cells thick, forming heavy networks of interlacing cells. The general features of the fibroblastic growth were similar in different tumors, although minor variations were noted in the looseness or density of the pattern.

MALIGNANT EPITHELIAL TUMORS

A squamous cell carcinoma of branchiogenic origin (Fig. 7) produced an abundant fibroblastic proliferation (Fig. 8). Epithelial sheets (Fig. 9) were noted as early as the 4th day and continued to form thereafter. Every so often a small cluster of cells in the midst of a sheet, or along its periphery, would become whorled in arrangement, and more dense and hyaline in appearance, suggesting the formation of pearls (Fig. 10). Cells once forming these pearl-like structures were never seen to resume proliferation.

Glandular carcinomas produced, in addition to fibroblasts and macrophages, irregular cords and clusters of epithelial cells, as shown in Fig. 12. The epithelial cells derived from glandular carcinomas never formed acini. They showed an even greater tendency to liquefy the plasma than did the epithelial cells of benign tumors. Of 2 mammary carcinomas having a similar appearance in the hematoxylin-eosin preparations (Figs. 11, 13) one produced epithelial cells in culture and one did not (Fig. 14). Thus tumors which appear similar in the usual histologic section may behave differently *in vitro*.

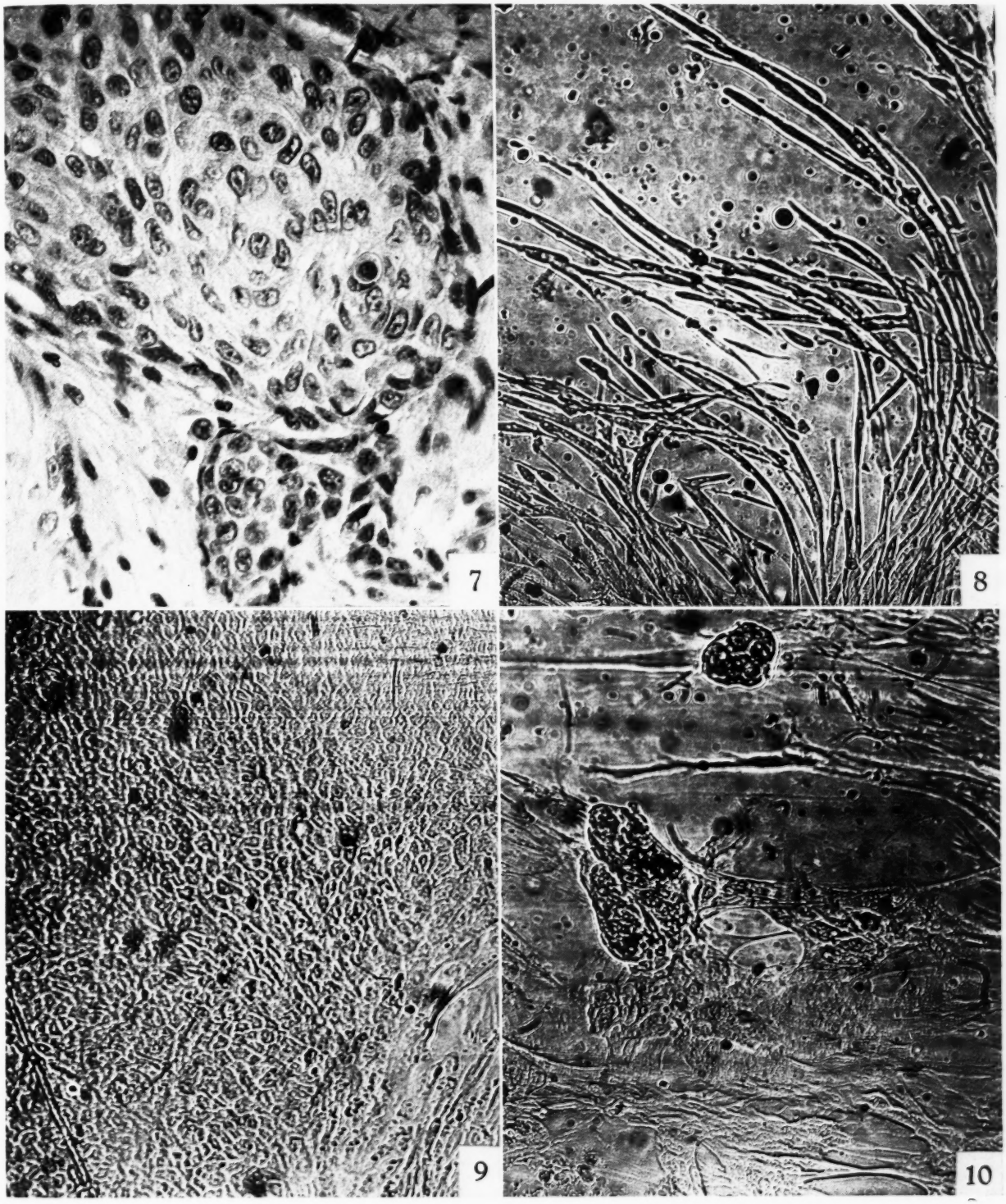


FIG. 7.—Hematoxylin and eosin stained section of a squamous cell carcinoma of branchiogenic origin, for comparison with the other photographs in the series showing the same tumor *in vitro*. Mag. × 440.

FIG. 8.—Fibroblasts *in vitro*, having proliferated from the stroma of the squamous cell carcinoma. Mag. × 113.

FIG. 9.—Squamous epithelial cells forming a large sheet *in vitro*. Mag. × 113.

FIG. 10.—Structures resembling pearls, formed *in vitro* by epithelial cells from the squamous cell carcinoma. Mag. × 113.

Not infrequently single epithelial cells broke away from the small nests and clusters, and such cells displayed ameboid motion. Proliferation of these isolated cells sometimes formed new colonies. This ability of the isolated neoplastic cell to progress by ameboid movement should be further studied, particularly in its possible relationship to invasion and metastasis. The cohesiveness of the cells should also be investigated to determine, if possible, the means by which a single cell breaks away from the cluster of which it is a unit.

No significant differences were noted in the behavior of primary and secondary tumors.

Benign Fibrous Connective Tissue Tumors

Vigorous fibroblastic proliferation occurred in cultures of leiomyofibromas of the uterus (Figs. 15, 16). The fibroblasts soon built up heavy meshworks around the explants. Intermingled with the fibroblasts were less numerous cells of a broader, flatter, though elongated form, which possibly were smooth muscle cells. This type of growth continued for several months of culture with no significant changes in the pattern.

Lymphosarcoma

Lymphoblastic cells were produced in abundance during the first 24 hours. This continued in some instances for several weeks. By this time fibroblasts had also grown out, and in 3 of the 4 tumors cultured the fibroblasts eventually dominated the growth while the lymphoid elements gradually disappeared. The remaining case, a retroperitoneal tumor (Fig. 17), showed a quite different sequence of events. From the very first, the emigrating cells were of the monocyte or macrophage type, as shown in Fig. 18. Very few cells were recognized as lymphoblasts. The monocytes continued to proliferate for more than a month, many of the cells wandering out of the plasma clot and attaching themselves to the inner surface of the test tube. Few fibroblasts grew.

Much discussion has taken place over the histogenesis of the lymphoblastomas and the matter has never been satisfactorily clarified. It would seem probable from these preliminary observations that new information might be acquired by investigating the lymphoblastomas in tissue culture.

Angiosarcoma

This tumor was obtained at autopsy and planted in tissue culture 27 hours after the patient's death. In spite of this, excellent growth was obtained. The tissue selected (Fig. 19) was from a metastasis in a rib. The angiomatous character of the tumor was more striking *in vitro* than in the hematoxylin-eosin section of the original tissue. Tubular structures (Fig. 20) were formed, appearing on the 3rd day and continuing for 6 months of culture. They seemed to grow as solid cords which later became hollowed out into true tubes, increased in size, and frequently formed lateral branches (Fig. 21). These vascular structures were composed of closely approximated endothelial cells which grew vigorously from the start and continued so until the cultures were discarded.

Melanosarcoma

Two melanosarcomas were cultured, both from metastases in the liver. Growth was poor. The only cells which emigrated in any number were macrophages and fibroblasts. The macrophages sometimes contained particles of the dark amorphous pigment and were the only emigrating cells which did contain pigment. Both tumors were obtained at autopsy and perhaps growth was poor because of autolysis. One of the cultured tumors developed a latent infection which ultimately destroyed it.

DESCRIPTION OF FIGURES 11 TO 18

Fig. 11.—Hematoxylin and eosin stained section of a mammary carcinoma. Mag. × 113.

Fig. 12.—Epithelial cells *in vitro* derived from the mammary carcinoma in Fig. 11. The epithelial cells form irregular clusters. Mag. × 113.

Fig. 13.—Hematoxylin and eosin stained section of a mammary carcinoma, similar in structure to the carcinoma in Fig. 11. Mag. × 113.

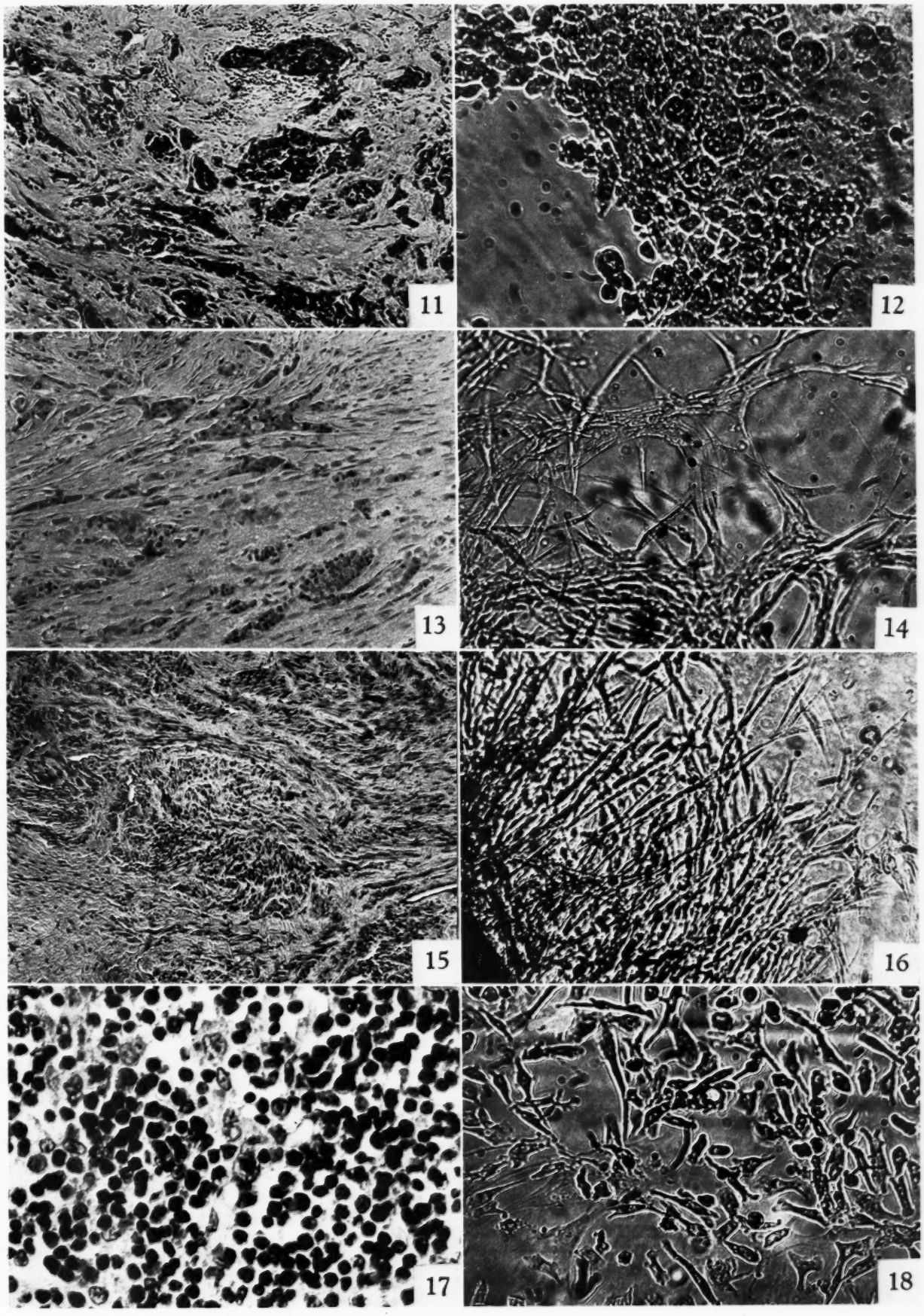
Fig. 14.—Tissue culture of the carcinoma in Fig. 13. No epithelial cells are present. The growth consists largely of fibroblasts. Mag. × 113.

Fig. 15.—Hematoxylin and eosin stained section of a leiomyofibroma of the uterus. Mag. × 113.

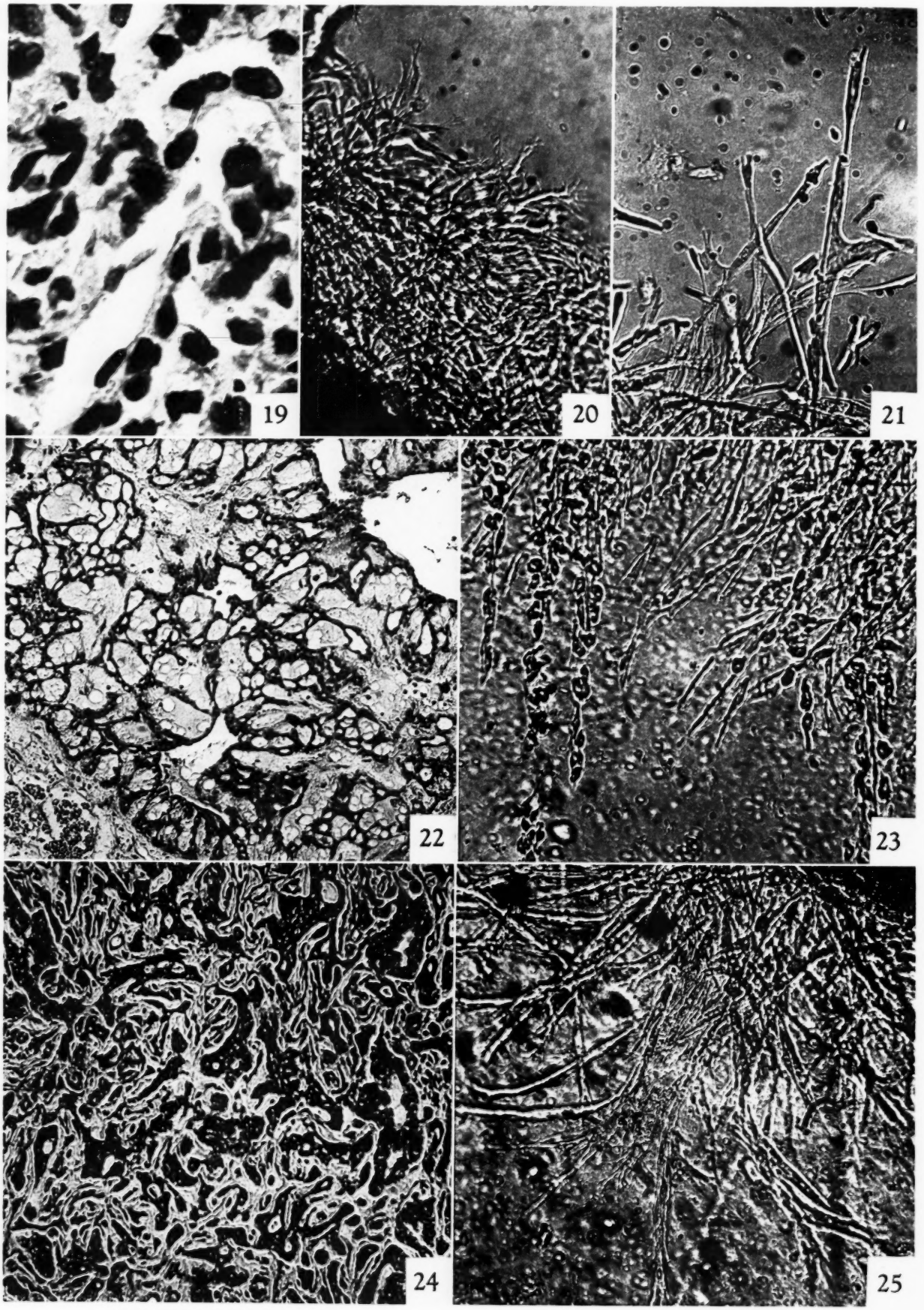
Fig. 16.—Tissue culture of the tumor shown in Fig. 15. The growth consists largely of fibroblasts. In the right lower corner of the photograph are somewhat broader and flatter cells which may be smooth muscle. Mag. × 113.

Fig. 17.—Hematoxylin and eosin stained section of a lymphoblastoma of retroperitoneal origin. Mag. × 440.

Fig. 18.—Macrophages *in vitro*. These were the only cells produced in tissue culture by the tumor shown in Fig. 17. Mag. × 113.



FIGS. 11-18



FIGS. 19-25

Mixed Tumor of the Parotid

Two mixed tumors of the parotid (Figs. 22, 24) were cultured. Only slight epithelial proliferation occurred and was never a prominent feature in the growth pattern. The only other cells produced in the cultures were macrophages and fibroblasts. The fibroblastic outgrowth from these 2 tumors, however, was of considerable interest. In hematoxylin and eosin stained preparations the 2 tumors were very similar, whereas in culture they proliferated in a strikingly different manner. In one case the outgrowing cells were small, delicate, and filamentous with rounded bulges produced by the nuclei (Fig. 23). These cells formed lace-like fragile filaments and were quite unlike any fibroblastic growth encountered in any of the other tumors of the series. The fibroblasts from the other mixed tumor were of the more usual type and differed in no essential way from the stromal outgrowth of other tumors (Fig. 25). The significance of this difference in the appearance of the fibroblastic pattern was not determined.

SUMMARY AND CONCLUSIONS

Various human neoplasms have been cultured by the roller tube method and their patterns of growth have been described and photographed. These tumors included carcinomas, sarcomas, and various benign growths.

Benign glandular epithelial tumors formed acini after a month of culture, whereas malignant epithelial tumors never formed acini.

A squamous cell carcinoma produced structures *in vitro* resembling epithelial pearls.

Individual neoplastic epithelial cells frequently broke away from the cell clusters and diplayed ameboid movement. Such isolated cells sometimes gave rise to new colonies. The possible significance of this in relation to the mechanism of invasion and metastasis is mentioned.

Lymphosarcomas produced lymphoblasts in 2 instances and only macrophages in a third. This suggested a possible heterogeneity in the histogenesis of the lymphoblastomas.

An angiosarcoma formed endothelial tubes *in vitro*. These structures resembled vessels and produced lateral branches.

Some neoplasms that presented a similar appearance in histologic sections behaved differently *in vitro*. These differences were noted both in the neoplastic cells themselves and also in the cells of the stroma.

The feasibility of the roller tube method for the study of a wide variety of living human neoplasms is demonstrated and its value as a method in investigative oncology emphasized.

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DESCRIPTION OF FIGURES 19 TO 25

Fig. 19.—Hematoxylin and eosin stained section of an angiosarcoma obtained at autopsy from a metastasis in a rib. Mag. × 1050.

Fig. 20.—Tissue culture of tumor shown in Fig. 19. Note tubular structures with branching ends. Mag. × 113.

Fig. 21.—Several large tubules composed of endothelial cells, derived from the angiosarcoma shown in the preceding photographs. Lateral branching is evident. Mag. × 113.

Fig. 22.—Hematoxylin and eosin stained section of a mixed tumor of the parotid gland. Mag. × 113.

Fig. 23.—Tissue culture of the tumor in Fig. 22 illustrating the peculiar appearance of the fibroblasts. Delicate filaments with bulging nuclei are formed. Mag. × 113.

Fig. 24.—Hematoxylin and eosin stained section of a mixed tumor of the parotid. This is not the same tumor as shown in Fig. 22. Mag. × 113.

Fig. 25.—Tissue culture of the tumor shown in Fig. 24. The growth consists of fibroblasts having an appearance and pattern quite different from those shown in Fig. 23. Mag. × 113.

Agent of Fowl Leukosis in Tissue Cultures*†

L. Doljanski, M.D., and M. Pikovski

(From the Cancer Laboratories, Department of Experimental Pathology, The Hebrew University, Jerusalem)

(Received for publication February 19, 1942)

The experiments reported in this paper were undertaken in order to determine whether cultivation of the agent of fowl leukosis *in vitro* in the presence of normal cells is possible. For this purpose investigations were conducted on: (a) normal cells cultivated in plasma from leukotic fowls, and (b) normal cells infected with leukotic agent and cultivated in normal plasma. The morphological aspect of the cell colonies was studied and their pathogenicity tested at regular intervals.

Previous attempts to cultivate the agent of fowl leukosis in the presence of normal cells have been reported as unsuccessful. Thus Furth and Breedis (4) indicated negative results in experiments on the cultivation of viruses producing leukosis ("pure" virus 1 and "complex" virus 13) in contact with proliferating normal cells. Ruffilli (8) found that normal bone marrow and normal fibroblasts, cultivated in leukotic plasma, produced no leukosis after inoculation into chickens.

MATERIAL AND TECHNIC

Source of the leukotic agent.—The experiments were carried out with the agent of chicken hemocytoblastosis, strain T1, obtained through the courtesy of Dr. Engelbreth-Holm in Copenhagen. Descriptions of this strain have been given (2).

The strain T1 of hemocytoblastosis, which is designated by Engelbreth-Holm and his coworkers as a pure leukotic strain, produces sarcoma according to Uhl, Engelbreth-Holm, and Meyer (9) in approximately one-third of the cases, when inoculated intramuscularly or subcutaneously. In our own experiments somewhat different results were obtained; in a total of 157 intramuscular inoculations of leukotic material so far carried out in this laboratory, 5 cases of local tumor were observed. Of these tumors, only 2 were pure spindle cell sarcomas, 2 contained large zones of hemocytoblasts in addition to spindle cells, and one had regressed. No case of sarcoma development was observed in a total of 41 intramuscular inoculations of active leukotic agent which was free of leukotic or normal blood cells (ultrafiltered agent maintained in cultures of normal spindle cells, *i.e.*, fibroblasts). The difference between the results obtained by Engelbreth-Holm and us may be due, among other causes, to changes undergone by this strain during approximately 100 passages carried out in this laboratory in the last 3 years.[1]

Leukotic plasma was obtained by centrifugalizing blood drawn from leukotic chickens at the height of the disease. Such plasma is almost always characteristically brownish red in color and coagulates either very slowly or not at all.

Ultrafiltrates of leukotic organs were prepared as follows: Heart and spleen of chickens which had died of hemocytoblastosis or had been killed in an advanced stage of the disease were removed, finely minced with scissors, and suspended in 5 parts of Ringer's solution. The suspension was kept in the refrigerator for an hour and then centrifugalized. The supernatant fluid was first passed through filter paper and then through a Berkefeld N filter.

Tissue cultures.—We worked with explants of bone marrow from normal adult chickens and of myocardium from adult chickens. In a number of experiments cultures of fibroblasts (1 to 4 passages) were used. The cultivation was generally carried out in Carrel flasks (models 3, 5) according to the standard technic (6). Chicken plasma diluted with Tyrode's solution in the proportion 1:2 was used as medium. A drop of diluted embryonic extract was added to the plasma for coagulation. Flask cultures were washed with Tyrode's solution and "fed" with heparinized plasma of normal chickens according to the method of Fischer and Parker (3).

Inoculations.—One- to 3-month-old chickens were used for inoculation. The contents of a Carrel flask were minced and suspended in Ringer's solution. The suspension was allowed to settle, the supernatant fluid was injected intravenously, and the residue intramuscularly. In some cases the cultures were overlayered with Tyrode's solution for 30 minutes before transfer; the supernatant fluid was drawn off, passed through a Berkefeld filter, and injected intravenously.

Histological technic.—Cultures in Carrel flasks were fixed in Helly's fluid, embedded in celloidin-paraffin, and cut in series. Sections 5 μ thick were stained by Giemsa's method.

EXPERIMENTAL

The pathogenicity of normal bone marrow and myocardium cultivated in plasma from leukotic chickens.—In these experiments bone marrow and myocardium of normal chickens were cultivated in the plasma of leukotic fowls. The virulence of the

* This investigation was aided by a grant from the Lady Tata Memorial Trust.

† A preliminary note was published in Nature, Lond., **146**: 302. 1940.

[1] In our recent cultural study with this strain (1), 4 of 68 chickens injected intramuscularly developed tumors at the site of injection. This was interpreted by the editors as indicating that the agent used produces both leukosis and sarcoma. In our opinion this conclusion is not warranted in view of the relative rarity and unclear genesis of these tumors.

plasma was always confirmed by inoculation into fowls. Experiments were repeated 4 times, with plasma from different leukotic chickens for each experiment. The cultures were "fed" with heparinized normal plasma after having been washed for half an hour with Tyrode's solution. Flasks treated in the same way, but containing no cell cultures, served as controls.

TABLE I: PATHOGENICITY OF NORMAL MYOCARDIUM AND NORMAL BONE MARROW CULTIVATED IN LEUKOTIC PLASMA

Flask No.	Tissue explanted	Cultivated, days	Chicken inoculated, No.	Result of inoculations	Lived after inoculations, days
		EXPERIMENT 1			
128	Bone marrow	14	1031	Pos.	14
133	" "	14	1032	Pos.	12
		EXPERIMENT 2			
386	Bone marrow	14	1310	Pos.*	60
			1311	Neg.	—
391	" "	22	1341	Neg.	—
			1342	Neg.	—
389	" "	35	1372	Neg.	—
			1373	Neg.	—
394	Myocardium	14	1312	Neg.	—
			1313	Pos.	17
396	"	22	1344	Neg.	—
395	"	35	1374	Neg.	—
		EXPERIMENT 3			
454	Bone marrow	15	1380	Neg.	—
			1381	Neg.	—
456	" "	21	1390	Pos.	32
			1391	Neg.	—
459	Myocardium	15	1382	Neg.	—
			1383	Neg.	—
460	"	21	1392	Neg.	—
			1393	Pos.	30
		EXPERIMENT 4			
604	Myocardium	15	1483	Neg.	—
			1484	Neg.	—
602	"	29	1510	Pos.	22

* This chicken also had a tumor (hemocytoblastoma with spindle cell sarcoma) at the site of inoculation.

After 14 to 35 days' cultivation the contents of the Carrel flasks were inoculated into chickens. The results are summarized in Table I.

The outcome of these experiments was inconstant. Of 7 cultures of bone marrow in leukotic plasma inoculated, positive results were obtained with 4 (3 with a 2 weeks' culture and one with a 3 weeks' culture). Of 7 cultures of normal myocardium in leukotic plasma, inoculated into chickens, positive results were obtained with 3 (one with a 2 weeks' culture, one with a 3 weeks', and one with a 4 weeks' culture). The control flask inoculations were always negative.

In spite of the inconstancy of the results these experiments justify the conclusion that, in the presence of normal cells, the agent of fowl leukosis can be maintained *in vitro* for a period of up to 29 days. The maintenance of the agent can succeed, not only in the presence of cells from the bone marrow but also in the presence of common spindle cells (fibroblasts) derived from myocardium.

The maintenance of the leukotic agent in cultures of normal fibroblasts.—In these experiments fragments of cultures of fibroblasts from the myocardium of adult fowls, were kept for 1 hour in ultrafiltrates of leukotic organs, planted in Carrel flasks, and cultivated for periods up to 38 days. Normal plasma diluted 1: 2—with Tyrode's solution in some experiments and with ultrafiltrates of leukotic organs in others—was used as culture medium. All cultures were fed with heparinized normal plasma after having been washed with Tyrode's solution.

After 15 to 38 days' cultivation, the cultures were inoculated into chickens. The results may be seen in Table II.

The agent of fowl leukosis brought into contact with cultures of fibroblasts derived from normal myocardium remained active as long as the cells were cultivated *in vitro*. Of 18 cultures which were inoculated after 2, 3, and 4 weeks' cultivation, 14 proved positive.

As may be seen from Table II the results following inoculation with cultures grown in plasma diluted with ultrafiltrates of leukotic organs were better than with those that had been cultivated in plasma and Tyrode's solution only. This may be due to the fact that the former were more heavily infected through the addition of ultrafiltrates to the medium. It is possible, however, that in the maintenance of leukotic agent the growth rate of the cell colonies plays a part: In media containing organ filtrates the cultures proliferate incomparably more actively and usually reach a much larger size than those grown in plasma diluted with Tyrode's solution. The relationship between the growth rate of the cell colonies and their ability to maintain the agent of fowl leukosis is the subject of our present investigations.

Experiments were undertaken subsequently to determine how long the agent of chicken leukosis can survive in the absence of living cells under the above experimental conditions. Carrel flasks were set up with a mixture of ultrafiltrates of leukotic organs and normal chicken plasma, and kept in the incubator. After varying periods the contents of the flasks were inoculated. Under these conditions the leukotic agent lost its activity within 24 hours.

The propagation of leukotic agent in cultures of normal fibroblasts kept in prolonged serial cultivation.—In these experiments fragments of fibroblast cultures derived from the myocardium of adult normal chickens were planted in Carrel flasks after having been kept for an hour in ultrafiltrates of leukotic organs. Normal chicken plasma diluted with Tyrode's solution served as medium. After 4 weeks' growth the cultures were transferred to a medium consisting of normal plasma diluted with Tyrode's solution. Further passages were carried out at intervals of 2 to 3 weeks, using the same medium throughout. The cultures were "fed" with heparinized normal plasma after having been washed with Tyrode's solution.

From the first passage on, such cultures proliferated vigorously. Though growth-stimulating extracts, except for the drop of very dilute embryonic extract necessary for coagulation, were not added to the medium, the cultures often attained within 7 days a diameter of 1 cm., a size usually found only in stimulated cultures.

TABLE II: PATHOGENICITY OF CULTURES OF NORMAL FIBROBLASTS PREVIOUSLY INFECTED WITH LEUKOTIC AGENT

(a) Planted in Normal Plasma Diluted with Tyrode's Solution

Flask No.	Cultivated, days	Chicken inoculated, No.	Result of inoculation	Lived after inoculation, days
		EXPERIMENT 1		
836	15	1723	Pos.	41
		1724	Pos	11
837	21	1731	Pos.	15
835	29	1739	Neg.	—
		EXPERIMENT 2		
859	16	1785	Pos.	18
		1814	Neg.	—
		EXPERIMENT 3		
868	15	1806	Neg.	—
883	16	1818	Pos.	13
		1819	Pos.	17
884	30	1834	Pos.	35*
869	38	1837	Neg.	—

(b) Planted in Normal Plasma Diluted with Ultrafiltrates of Leukotic Organs

		EXPERIMENT 4		
839	15	1721	Pos.	14
		1722	Pos.	25
838	21	1732	Pos.	33*
840	29	1737	Pos.	16
		1738	Pos.	12
		EXPERIMENT 5		
862	16	1786	Pos.	18
		1787	Pos.	21
860	31	1816	Pos.	21
		1817	Pos.	16
		EXPERIMENT 6		
870	15	1808	Neg.	—
		1809	Pos.	35
871	38	1839	Pos.	27
		1840	Pos.	13
		EXPERIMENT 7		
886	16	1820	Pos.	46
		1821	Pos.	18
885	30	1835	Pos.	16
		1836	Pos.	16

* Anemic forms of leukosis.

TABLE III: PATHOGENICITY OF COLONIES OF NORMAL FIBROBLASTS INFECTED WITH LEUKOTIC AGENT AND KEPT IN PROLONGED CULTIVATION

Flask No.	Passage, No.	Cultivated, days	Chicken inoculated, No.	Result of inoculation	Lived after inoculation, days
(a) Results of Intramuscular Inoculation of Cell Cultures					
884	1	30	1834	Pos.	35*
925/8	2	44	1860	Pos.	21
			1861	Pos.	38
939/41/2	3	60	1931	Pos.	35
975/8	4	74	1973	Pos.	19
			1974	Pos.	14
993/4	5	86	2021	Pos.	9
			2022	Pos.	10
1100/1	10	172	2126	Neg.	—
			2127	Pos.	15
1105	11	178	2134	Pos.	21
(b) Results of Intravenous Injections of Ultrafiltrates of Supernatant Fluid					
884	1	30	1832	Pos.	52*
			1833	Pos.	32
925/8	2	44	1858	Pos.	15
			1859	Pos.	22
939/41/2	3	60	1933	Pos.	12
			1934	Pos.	15
975/8	4	74	1970	Pos.	11
			1971	Pos.	14

* Anemic forms of leukosis.

After various periods the cultures were inoculated into chickens. In some cases the cultures were washed for 30 minutes with Tyrode's solution before transfer, and the ultrafiltrate of the supernatant fluid was injected intravenously. Table III summarizes such an experiment.

A prolonged maintenance of the agent of fowl leukosis was obtained when fibroblasts which had been infected with this agent were cultivated through successive passages. We have been able so far to maintain the leukotic agent in cell cultures for 178 days. Not only the cell cultures but also the ultrafiltrates of the fluid in which the cultures were washed have proved infective.

The leukotic agent is not attenuated in culture: The percentage of takes after prolonged cultivation is not reduced; the duration of the illness in chickens inoculated with cultures of the later passages is as usual; and the clinical and histological picture remains that of classical hemocytoblastosis (Figs. 1 and 2).

Microscopic examination of cultures infected with the agent of fowl leukosis showed that it caused no changes in their aspect, the nuclear and cytoplasmic structure of individual fibroblasts remaining indistinguishable from the normal. The leukotic cultures, like those of normal fibroblasts, did not liquefy their plasmatic medium.

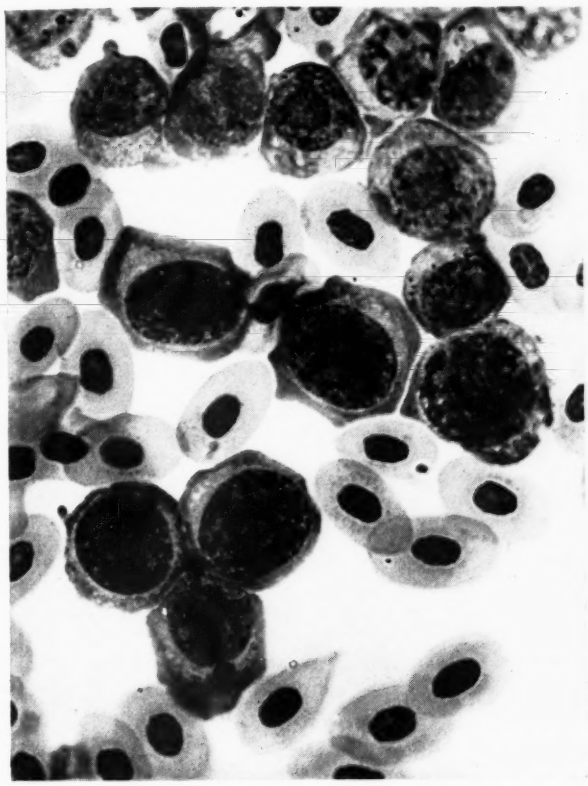

FIG. 1.—Blood of chicken No. 2127 infected with leukotic agent which had been cultivated 172 days *in vitro* (see Table III). May-Grünwald-Giemsa stain. Mag. × 1200.

In one case a tumor (hemocytoblastoma with spindle cell sarcoma) developed at the site of inoculation after the injection of bone marrow cultivated in leukotic plasma. In no case was a sarcoma found at the site of inoculation of leukotic agent which had been maintained in cultures of fibroblasts.

DISCUSSION

Up to the present the active agent of fowl leukosis has been successfully maintained *in vitro* only in cultures of tissues from leukotic fowl. Furth and Stubbs (5), Verne, Oberling, and Guérin (10), Ruffilli (7, 8), Furth and Breedis (4), and Doljanski and Pikovski (1) have shown that cells derived from the leukotic fowl can be cultivated *in vitro* without loss of pathogenicity for considerable periods of time. Chickens inoculated with such cell colonies develop typical leukosis.

In 1934 Furth and Stubbs (5) reported that cultures of sarcoma cells (strain 13, associated with erythroblastosis) remained highly virulent after cultivation *in vitro* for 67 days and produced sarcoma with or without erythroblastosis when inoculated into the fowl.

A brief report on the cultivation of bone marrow from leukotic chickens was published shortly afterward by Verne, Oberling, and Guérin (10). They found that blood cells rapidly disappear in leukotic bone marrow cultures and that transformation of the bone marrow culture into a pure fibroblast culture is already complete after the 4th passage. Some cultures inoculated after 18 days' cultivation were not pathogenic. In further experiments the same investigators added fragments of normal bone marrow to cultures of leukotic bone marrow in Carrel flasks. In a few inoculation attempts with such cultures there were takes with two 8-day- and one 18-day-old cultures.

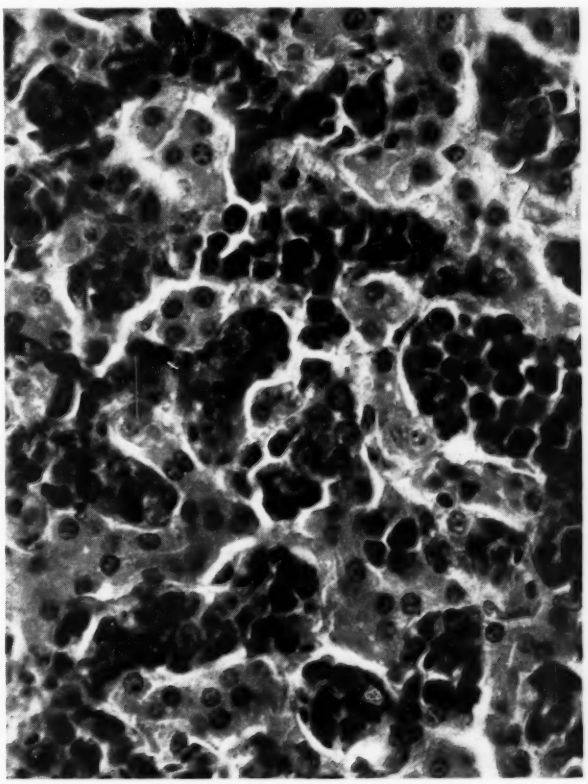

FIG. 2.—Liver of the same chicken. Hematoxylin and eosin stain. Mag. × 615.

An extensive investigation on the cultivation of bone marrow, spleen, and blood cells of leukotic fowls, and of tumors associated with leukosis and lymphomatosis, was reported in 1937 by Furth and Breedis (4). Leukemic myeloblasts, when incubated *in vitro* in a liquid medium, showed mitotic division for 26 days and retained throughout their ability to produce leukosis. The cultures of spleen and bone marrow remained pathogenic even after 56 days' cultivation *in vitro*. Bone marrow and spleen cultures proved infectious, however, only when the material used for cultivation was from a fowl with myeloblastic leukemia.

In this case the myeloblasts in cultures were found to persist "in symbiotic growth with fibroblast-like cells." On the other hand, cultures of bone marrow from chickens with erythroleukosis, which rapidly turned into pure fibroblast-like cultures, failed to produce leukosis. From the different behavior of cultures in the presence and absence of the blood cells the authors concluded that primitive blood cells are indispensable to the multiplication of the leukotic agent. "Oncogenic viruses multiply *in vitro* only in the presence of cells on which they confer neoplastic properties." This opinion is further supported by experiments with viruses 2, 12, and 13, which "stimulate" not only primitive blood cells but also endothelial cells or fibroblasts *in vivo* to unrestricted growth. These viruses, which Furth designated as "complex," can be maintained in cultures of tumor cells or of proliferating endothelial cells from leukotic fowls in the absence of blood cells, whereas virus 1, which is regarded by Furth as pure and which he believes has an exclusive affinity for primitive blood cells, can persist only in the presence of the latter.

In the same year Ruffilli (7) showed that cells derived from the myocardium of leukotic chickens remain pathogenic after repeated subculturing *in vitro*. He transferred such cell colonies through 44 passages (122 days) without loss of infectivity. The "leukotic" fibroblasts differed in certain respects from normal: They possessed notably developed mitochondria, abundant neutral red granules of irregular size, and they readily underwent transformation into ameboid cells.

Doljanski and Pikovski (1) reported experiments on the cultivation of bone marrow, blood cells, and myocardium of chickens with hemocytoblastosis (strain T1, Engelbreth-Holm). The cultures of leukotic blood cells underwent transformation into fibroblast colonies and the original blood cells disappeared almost completely. It was shown that such cultures remain infectious in all stages of their development, irrespective of the presence or absence of blood cells. Bone marrow cultures were also transformed in the course of cultivation into pure spindle cell colonies; nevertheless, they remained infectious. Also cultures of fibroblasts derived from the heart of leukotic chickens proved to be highly virulent. It has been possible to maintain such fibroblast colonies alive and infective *in vitro* for 181 days. Cultures of spindle cells from leukotic heart have the same architecture as pure cultures of normal fibroblasts. The "leukotic" fibroblast is in no way different from normal.

The experiments of Ruffilli (7, 8) and of Doljanski and Pikovski (1) show the probability that common fibroblasts originating from the leukotic organism are able to ensure the persistence of the leukotic agent of some strains *in vitro*. This conclusion could not, however, be regarded as definitely established. Neither the experiments of Ruffilli nor our own prove beyond doubt that maintenance of the leukotic agent can be assured by the presence of common fibroblasts alone. All cell cultures employed in these experiments were derived from leukotic tissues and the latter always contained leukotic blood cells. It is possible, although very improbable, that a few leukotic blood cells may have survived undiscovered among the proliferating fibroblasts, even after prolonged cultivation. The pathogenicity of these cell strains, therefore, may be the result not of persistence of the agent but of the survival of single leukotic cells.

For a definite solution of the question whether the leukotic agent can be maintained *in vitro*, and in order to ascertain whether common fibroblasts alone are capable of maintaining it, a new experimental approach was necessary. We have attempted, accordingly, to maintain the agent of fowl hemocytoblastosis in cultures of normal cells. The agent which we used for the infection of normal cells was derived from ultrafiltrates of leukotic organs and was certainly free from leukotic cells.

Our investigations prove that:—

Maintenance of the agent of fowl leukosis (hemocytoblastosis, strain T1 of Engelbreth-Holm) in the presence of proliferating normal cells is possible for as long as 178 days.

The agent does not require primitive blood cells for its maintenance. Possessing the property of stimulating primitive blood cells to unrestricted growth *in vivo*, it requires for its maintenance *in vitro* only the presence of proliferating common mesenchyme cells.

The experiments of Furth and his coworkers (4, 5) lend some support to the view that differences exist in the behavior of various strains of leukotic viruses *in vitro*, and that this difference may perhaps depend upon differences in their respective cell affinities. In view of this possibility caution is necessary in generalizing from our findings. These are valid for strain T1 of hemocytoblastosis which we used, but a final decision on the question whether ability to persist in the absence of common fibroblasts is a property of other leukotic viruses must await further investigation.

The leukotic agent retained its activity *in vitro* undiminished for almost half a year though the cultures were planted in fresh medium at each passage and washed before each transfer, and though only a small part of a colony was used for transfer. This fact shows clearly that a real increase of the agent of fowl leukosis occurs *in vitro*. In the absence of an adequate quantitative method for estimating the activity of leukotic agent, no conclusions as to the extent of this increase can as yet be drawn.

CONCLUSIONS

1. Bone marrow from normal fowls explanted in leukotic plasma and cultivated *in vitro* up to 3 weeks produces leukosis when inoculated into chickens.

2. Myocardium from normal fowls explanted in leukotic plasma and cultivated *in vitro* up to 4 weeks is also infective.

3. When cultures of normal fibroblast are infected with the agent of fowl leukosis (hemocytoblastosis, strain T1 of Engelbreth-Holm) and planted in normal plasma diluted either with Tyrode's solution or with ultrafiltrates of leukotic organs, the agent remains

active as long as the cells are cultivated (up to 38 days). In the absence of living cells the agent of fowl leukosis loses its activity within 24 hours.

4. By serial cultivation of fibroblasts infected with leukotic agent the latter could be maintained *in vitro* for 178 days.

5. Both the cell cultures and the cell-free supernatant fluid from the cultures proved infective for fowls.

6. The presence of leukotic agent in the cell cultures caused no changes in the appearance of the latter and no alterations in individual fibroblasts.

7. It is suggested that a real increase of leukotic agent occurs *in vitro*.

8. Intramuscular inoculation of leukotic agent in cultures of normal fibroblasts was in no case followed by the development of sarcoma at the site of inoculation.

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The Occurrence of Benign and Malignant Mammary Lesions in Rats Treated with Crystalline Estrogen*

Milton J. Eisen, M.D.

(From the Department of Cancer Research, College of Physicians and Surgeons, Columbia University, N. Y.)

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The relationship of ovarian hormones to the development of the breast and, logically, to mammary cancer appeared clearly outlined in the results of early castration experiments on mice. Prepuberty ovariectomy inhibited normal growth of the breast, with the consequence that females of strains with mammary tissue highly susceptible by heredity to spontaneous malignant alteration failed to develop this lesion. Nevertheless, later experiments indicated that this generalization needs modification, for Woolley, Fekete, and Little (48) demonstrated that the adrenal gland is capable of assuming the function of the ovary in animals spayed within 24 hours after birth.

Estrogens in mice.—With the demonstration that mammary cancer can be induced in animals by various estrogenic preparations, a firm foundation seemed assured for the theory that cancer of the breast is a local manifestation of excessive stimulation of this organ by one of the substances responsible for its physiologic control, or of an abnormal stimulation determined by some endocrine dyscrasia. Proof for these hypotheses has been sought for preeminently in mice. It quickly became apparent from the observations of Lacassagne (29), Gardner and his coworkers (18), Burrows (7), Cramer and Horning (9), Suntzeff and his associates (43), Bonser, Stickland, and Connal (6), Twombly (46), Taylor and Waltman (45), and others that treatment of mice with estrogens gave rise to a higher percentage of mammary cancers than would occur normally, and at an earlier age in females of stocks genetically susceptible to the disease in a greater or lesser degree. Males of these same strains, which if untreated did not have tumors of the mamma, also developed cancer if they received estrogen. In the absence of the hereditary factor, however, the female sex hormone induced no mammary cancers even in females. It is worthy of note that exceptions to the former sequence have been discovered. Thus, according to Bischoff and his coauthors (3) the mammary tissue of Marsh mice, though highly susceptible by heredity to the development of cancer, resisted the effect of injected estrogen. Excellent general reviews of much of the literature have been contributed by Lacassagne (30), Cramer (8), Gardner (17), Allen (1), and Loeb (31).

Estrogens in rats.—Species other than the mouse have been less widely employed to elucidate the hormonal mechanisms related to mammary cancer. The rat, rarely the host of spontaneous malignant tumors of the mamma, also proved resistant to excessive exogenous estrogenic stimulation in the experiments of Heiman and Krehbiel (28), Emge (14), Druckrey (10), Silvestroni (39), and Grumbrecht (26). McEuen (32), on the other hand, observed an occasional cancer of the mamma, among other neoplasms, in rats given estrogen and painted with tar. Mammary cancer developed more readily in animals that received implants of pellets of crystalline hormone, as would be concluded from the publications of Geschickter (21, 22), and Noble, McEuen, and Collip (36). Some of the tumor-like growths described by the latter investigators appeared not to have progressed beyond the stage of hyperplasia, since they lacked the quality of autonomy. Thus regressions occurred following extraction of the estrogen pellet as well as after the administration of progesterone (35).

Dependence of action of estrogens on milk factor.—The view that excessive or abnormal estrogenic action, instead of acting directly as a carcinogenic stimulus, may only increase the total mammary tissue or induce chronic mammary hyperplasias which in turn are likely to be susceptible to other agents primarily responsible for the malignant alteration, finds some justification in observations on the factor transmitted with the milk. Twombly (47) was able to produce mammary cancer in C57 black mice—a strain not susceptible to this disease either spontaneously or if treated with massive doses of estrogens—by nursing estrogenized males on females of the R III strain, which has a high frequency of spontaneous mammary carcinoma. Bittner (4), on the other hand, inhibited tumor development in estrogenized A mice—a strain in which the females are normally susceptible to spontaneous mammary cancer—through fostering by C57 black mice. These results were confirmed by Shimkin and Andervont (38). From these observations, therefore, the importance of estrogenic stimulation appeared subordinate to, or possibly conditioned by, a factor transmitted in milk.

Benign and malignant mammary lesions produced by carcinogenic hydrocarbons.—While endocrine factors indubitably are of considerable importance for the induction of mammary neoplasms, observations with the carcinogenic hydrocarbons prove that they are not indispensable in the initiation of either benign or malignant lesions of the breast. Statistical evaluation of tumor production in animals by the hydrocarbons offers additional support for the contention that the malignant change occurs as the result of factors operating locally, and that the type of tumor induced depends upon the production of a permanent modification in a given cell after chance contact with a specific irritant. The significance of the mammary alterations induced by the hydrocarbons becomes readily apparent in the light of these concepts. Thus Stewart (40) noted the local development of mammary cancer about methylcholanthrene in C3H mice, a strain that is highly susceptible to mammary cancer, while Strong and Smith (41), Strong and Williams (42), and Bonser and Orr (5) reported a similar effect in mice genetically resistant to the spontaneous occurrence of mammary neoplasms. Estrogens, nevertheless, appear to exercise some determining influence on the site of action of absorbed hydrocarbons, for Perry and Ginzton (37) observed a considerable number of cancers of the breast and uterus in mice given the hormone and painted on the skin with dibenzanthracene.

* Read at the 35th Annual Meeting of the American Association for Cancer Research, Boston, Massachusetts, March 31, 1942.

In rats and mice of known lineage, Dunning, Curtis, and Bullock (11) obtained a small number of mammary carcinomas about subcutaneous nodules of benzpyrene in paraffin. Later Dunning, Curtis, and Eisen (12) reported that methylcholanthrene possessed an even greater faculty of producing proliferative lesions in the breast of the rat. Proliferation of the columnar cells lining the ducts, squamous cell metaplasia of the hyperplastic ducts—transformed in a number of instances by continued function of the altered cells into squamous cell cysts—were found in males and females about foci of the carcinogen before and after the appearance of sarcoma. A small but not negligible number of animals developed mammary adenocarcinomas as a result of progressive action locally of the specific irritant without the intermediary squamous cell metaplasia, while in others squamous cell cancer developed from the metaplastic ducts or cysts.

Relation of absorbed hydrocarbons to mammary cancer.—A number of experiments, such as those of Maisin and Coolen (33), Mider and Morton (34), and Engelbreth-Holm (15), indicate that methylcholanthrene absorbed from a solution in benzol applied to the skin, especially if different areas are used successively to avoid cutaneous carcinogenesis, may assist the development of mammary cancer in genetically susceptible mice. The resulting increase in the number of mammary tumors, or reduction in the average age at which they occur, is perhaps an expression of the local action of absorbed carcinogen on areas of mammary hyperplasia commonly known to precede malignant alterations in such strains.

Table I: Quantities of Crystalline Estrogen Administered to 134 Rats, either in a Single or in Divided Doses

Dose, mgm.	1	2–5	6–10	11–15	16–20
Number of rats	26	25	45	23	15

Table II: Number of Rats Which Died in Each Month after the Administration of Crystalline Estrogen

Time after implantation, months	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Number of rats alive	134	134	130	129	127	124	120	117	103	88	73	66	54	40	36	32	26	19	15	12	9	8	6	5	4	2	2	1
Number of rats dead	0	4	1	2	3	4	3	14	15	15	7	12	14	4	4	6	7	4	3	3	1	2	1	1	2	0	1	1

The following paragraphs describe the results of an extensive series of investigations on the effect of slowly absorbed crystalline estrogen upon the mammary tissue of rats of known genetic constitution.

EXPERIMENTAL

The rats employed were members of the inbred Sherman strain, with the exception of a small number of animals of several unrelated inbred families. Spontaneous mammary lesions resembling those occurring in rats that received female sex hormone have not been observed in untreated rats. While fibroadenoma of the mamma has arisen occasionally in old, untreated Sherman females, a sufficient number of such neoplasms has not been collected to ascertain whether the incidence is significantly higher than in comparable members of other strains. Certainly the equal susceptibility of both sexes to exogenous estrogen can be accepted as corroborative evidence of the dependence of the mammary alterations on estrogenization, for spontaneous changes in the breast are practically nonexistent in the male. The animals were maintained on the standard laboratory ration of equal parts Purina dog chow and Rockland rat diet with water as desired, supplemented by a liberal supply of fresh carrots and 6 drops of cod liver oil once weekly, a regime which has proved adequate for the growth and reproduction of normal rats.

The hormone employed, estradiol dipropionate,[1] was administered to animals from 3 to 5 weeks of age. An early technic—subcutaneous implantation of weighed amounts of crystalline material through a small skin incision—employed in a few animals only, was superseded by the injection of the hormone dissolved in liquefied paraffin (melting point 48° C.) in a concentration of 5.0 mgm. per 0.1 cc. The warm paraffin, injected subcutaneously, intramuscularly, or intraperitoneally, solidified as firm ellipsoids from which absorption of estrogen occurred slowly and continuously (13). In performing the subcutaneous implantations no deliberate attempt was made to place the material in the mamma. As judged by the vaginal smear, or the position and functional state of the testes, the average time required for the absorption of 5.0 mgm. was 151 ± 5.9 days. A minimal foreign body reaction developed about the nodules. They eventually became encysted in a relatively acellular connective tissue which in no instance showed evidence of sarcomatous transformation. With the exception of those animals that received a single dose of 1.0 mgm. and no further treatment, all were given supplementary quantities of 2.5 or 5.0 mgm. when the vaginal smear or condition of the testes indicated cessation of absorption from the earlier implanted foci. The latter rats therefore remained under the constant influence of exogenous estrogen during their entire lives. For this study 134 animals were used, of which 63 were females and 71 males.

In Table I the rats are grouped according to the amount of estrogen administered, which varied from 1 to 20 mgm. in divided doses. Table II shows the number which died in each month following the initial implantation. Although considerable variation exists in the rate of utilization of identical amounts of estrogen in different rats, no consistent correlation was discerned between the rapidity of absorption and the extent of the associated lesions. Thus the length of time for continuous action of effective quantities of exogenous estrogen plays an important role in determining the nature of the induced alterations. Therefore all rats are grouped together regardless of the total divided dosage.

[1] Estradiol dipropionate was generously furnished by Dr. R. MacBrayer, Ciba Pharmaceutical Products, Inc., Summit, N. J.

Mammary Lesions

As the object of these experiments was primarily the investigation of mammary alterations, these will be described in greatest detail. Mammary tissue representative of successive stages of constant influence by estrogen was secured by biopsy when not made available through the death of animals. The organ was equally influenced throughout, and the absence of more extensive local changes about hormone implanted in it by chance attested the generalized nature of the estrogenic influence. As the type of lesion encountered depended largely upon the duration of abnormal estrogenic action, definite architectural patterns constituted in general the outstanding morphologic characteristics of successive stages in a continuously developing alteration determined by a constantly available agent. Nevertheless, this division into stages proved somewhat arbitrary in that the presence of the dominant lesion corresponding with a certain interval did not exclude necessarily the possible discovery of areas characteristic of earlier or later reactions. The alterations, as they were continuous, evolved without clear demarcation through successive periods.

Proliferation of duct epithelium.—Although some details pertaining to the ultimate nature of the hormonal control of mammary growth and function have not yet been completely elucidated, many facts rest upon a firm experimental foundation. In addition to physical factors and pituitary stimuli the 2 hormones elaborated in the ovary, estrogen and progesterone, are of prime importance. While no claims can be advanced for a strict delimitation of their effects, estrogen functions primarily as a stimulator of duct development, and progesterone, when acting in conjunction with the former, as a motivator of acinar growth and possibly of function. Proof of these contentions is made readily available by a study of gross mounts of the breast and corroborative histologic preparations, as is known from numbers of reports in the literature and as can be adduced from the present series of animals.

A considerable degree of diffuse proliferation occurred in the breast as early as 26 days after the implantation of crystalline estrogen. Microscopically the prominent ducts were evident immediately, as a consequence of hyperplasia of the columnar lining cells. Instead of the 1 or 2 layers of resting cells normally found at this age, considerable areas of the ductal lumina were filled with proliferating cells, well confined, however, by the basement membrane. Mitotic figures, although common, presented no evidence of being atypical. Acinar development was minimal or absent (Fig. 1).

Secretion.—After approximately 40 to 60 days, when the primary proliferative stage had attained its zenith, signs of functional activity appeared in the duct epithelium. Instead of the profuse aggregation of elements characteristic of the earlier stage there was now an arrangement of the cells in several orderly layers. Secretory vacuoles appeared in the cytoplasm and eventually, after having been extruded or desquamated into the lumina together with cellular fragments, coalesced to form pale eosinophilic masses.

At the height of the diffuse secretory phase induced by crystalline estrogen, gross mounts offered striking proof of the generalized growth of the mamma, particularly evident in the ducts. The great increase in the number of branchings and in the caliber of the tubules contrasted strongly with the relatively undeveloped duct system of untreated control females and the rudimentary mammary elements of normal male rats.

Duct dilatation.—This logical development occurred at 60 to 90 days as a result of the continued activity of the secreting cells and retention of the collected secretions. For the function induced by an abnormal hormonal concentration, in the absence of other factors required for lactation, was necessarily abortive and the elaborated fluid, after stagnating, produced enlargement of the lumina.

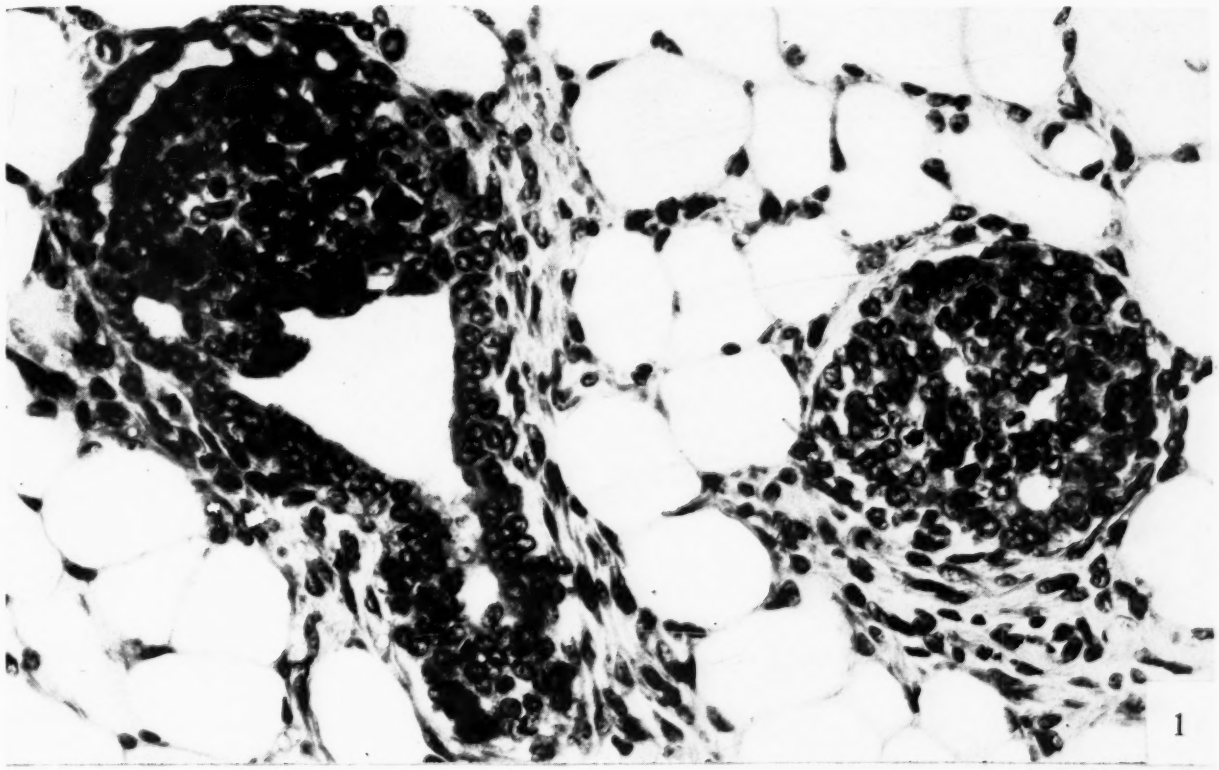
Duct cystosis.—This more advanced development of the former stage, the formation of poorly defined but fairly well localized cystic zones visible macroscopically, at times 0.5 to almost 1.0 cm. in diameter and containing a thick fluid comparable in consistency with milk, was attained at 90 to 150 days. Flattening and desquamation of the lining cells accompanied large accumulations of cystic fluid, probably as a result of cellular disintegration consequent on mounting intracystic pressure. The cystic zones displaced the fat cells normally present between mammary lobules.

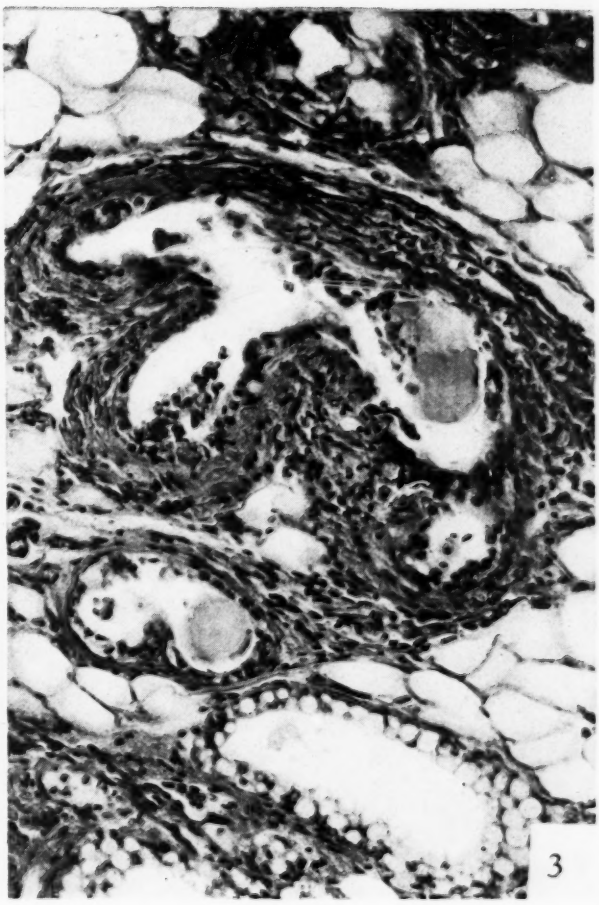
DESCRIPTION OF FIGURES 1 TO 3

Fig. 1.—Female. Proliferative lesions of early stage in mammary ducts. Estradiol dipropionate, 3.0 mgm., 32 days. Mag. × 375.

Fig. 2.—Female. Stage of diffuse functional activity in mammary ducts, in which collected secretions produce dilatation and cystic zones. Note the almost complete displacement of the fat cells. Estradiol dipropionate, 6.0 mgm., 132 days. Mag. × 55.

Fig. 3.—Female. Fibrosis about enlarged ducts. Note the flattened, inactive epithelium lining the spaces, the inspissated secretions containing desquamated cells, beginning distortions of the ducts, reappearance of fat cells, and the epithelium of some ducts still in the stage of secretion. Estradiol dipropionate, 17.5 mgm., 387 days. Mag. × 187.5.





FIGS. 1-3

The progressive but overlapping alterations consisting of secretion leading to duct cystosis are illustrated in Fig. 2.

Fibrosis.—Stimulated growth of the periductal connective tissue, followed by increasing contractions and distortions of the previously enlarged ducts, became evident at 150 to 180 days. Varying intensities of these alterations persisted in animals that survived longer. Purely mechanical factors associated with outward pressure of the overdistended ducts, or chemical irritation resulting from dissipation of the secretions or products of their decomposition, possibly accounted for the fibrosis of progressively increasing severity. The condition of the surviving epithelium, now apparently in a stage of relative passivity, appeared to be secondary to the development of the lesions in the surrounding stroma. The new connective tissue, at first sparse and cellular, became transformed later into hyalinized bundles. Physical alterations in the masses of connective tissue gave rise to bizarre distortions of the enlarged duct spaces, including intracystic intrusions, especially when viable proliferating or secreting cells persisted in the epithelial layers (Fig. 3).

The fibrosis eventually became so diffuse as to displace largely the fatty tissue which had reappeared after the initial development of contraction of the enlarged or cystic ducts. These now persisted as more isolated, irregularly shaped spaces while in some instances their dimensions were reduced further to that of shrunken but elongated tubes. The remaining epithelial cells, usually arranged in single layers, gave the impression of having undergone extensive regressive changes. Nevertheless, in some areas they appeared indistinguishable from normal resting cells. Foci of round cells and macrophages were encountered not uncommonly in the stroma.

The more advanced condition (Fig. 4) and the well developed fibrotic mammary lesion (Fig. 5) do not differ significantly from certain forms of chronic cystic mastitis observed in the human breast. In some instances the fibrosing process (Fig. 6) may eventually give rise to a more intimate type of sclerosing adenomatosis by producing dissociation of individual mammary tubules. This likewise is the counterpart of a chronic benign mammary proliferation occurring in the human subject.

While on the whole the different stages appeared to follow in sequence as part of a continuous process, it is necessary to emphasize that not all animals maintained for comparable periods under the constant influence of slowly absorbed estrogen showed identically advanced lesions, nor were different areas of the mamma of a single animal equally affected.

Late secondary epithelial proliferation.—Lesions of this type were observed in 6 animals, dead or sacrificed after 242 or more days. Unquestionably some stage of the chronic fibrotic lesion with persisting although altered ducts and irregular cystic formations constituted the outstanding mammary alteration found in these rats. In addition, irregularly distributed in these lesions were found foci, in some cases multiple, of epithelial cells again presenting evidence of proliferative vigor. As they were arranged in several layers they caused either partial or complete obliteration of the lumina into which they grew. They were identified readily by the deeper stain invariably assumed by their nuclei. The formation of secondary glands in the solid epithelial masses was common, and mitotic figures were encountered frequently. Nevertheless the cells showed no evidence of the disorder characteristic of neoplasia, and invasion of the surrounding stroma did not occur. Localized zones of papilliferous proliferation of larger, somewhat eosinophilic cells have been observed occasionally, but in no instance could these cells be compared directly with the so called pale epithelium of human chronic mastitis.

Fig. 7 is a longitudinal view of a large, originally cystic duct with imposing growth of the lining cells. In Fig. 8 this type of proliferation is illustrated in a number of ducts.

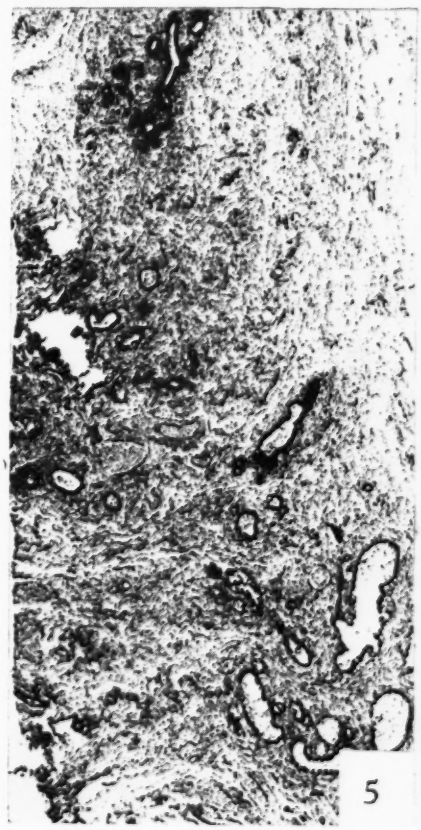
Malignant alteration.—Lesions, interpreted as carcinoma of the breast because of the massiveness of the process, its invasive qualities, and the attendant histologic signs commonly accepted as denoting malignant disease, were found in 2 rats. In one the zone of neoplastic change was discovered microscopically in an animal with a chronic fibrotic mammary lesion, dead 376 days after the first implantation of estrogen. The growth (Fig. 9) is a well differentiated adenocarcinoma which appears to have originated in an area characteristic of the earlier chronic benign lesion.

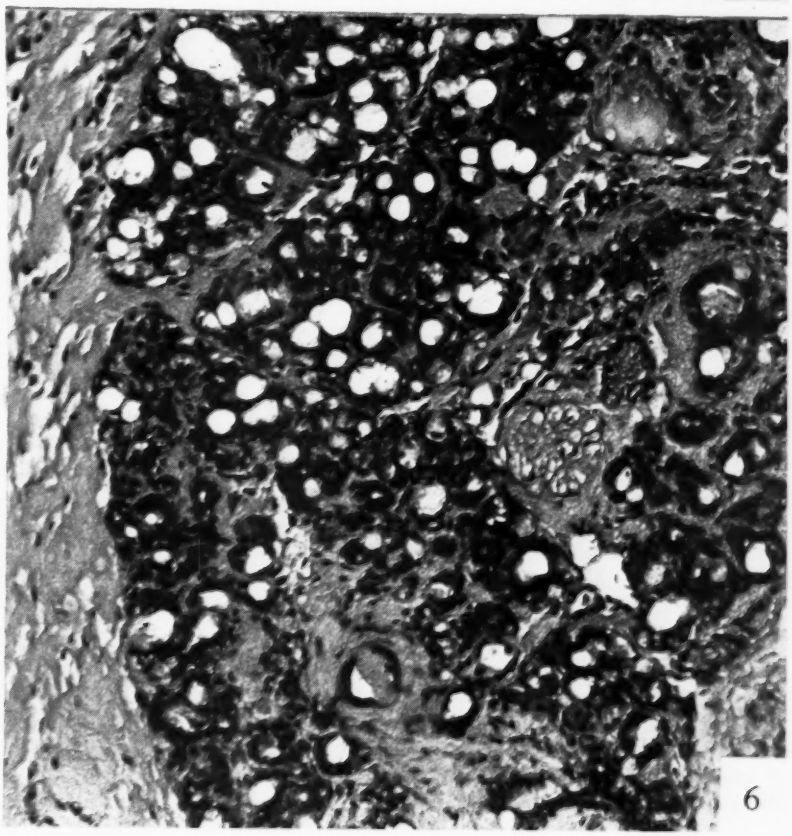
DESCRIPTION OF FIGURES 4 TO 6

Fig. 4.—Female. More extensive fibrosis about a large cyst, with many irregularly shaped contracted ducts in the abundant stroma. The tissue is from the same animal as that illustrated in Fig. 3. Mag. × 90.

Fig. 5.—Male. Advanced fibrosis of the mammary tissue. Note the extensively developed fibrous tissue containing remnants of enlarged, distorted ducts and contracted epithelial spaces, and the displacement of the fat. Estradiol dipropionate, 10.0 mgm., 181 days. Mag. × 35.

Fig. 6.—Female. Sclerosing adenomatosis of the breast produced by advanced fibrosis dissociating individual, contracted mammary tubules. Estradiol dipropionate, 12.5 mgm., 551 days. Mag. × 200.





FIGS. 4-6

The second animal with cancer, sacrificed after 803 days, had 4 circumscribed subcutaneous masses 2 to 3 cm. in diameter. The mamma was diffusely involved by benign fibrocystic disease. Histologically the neoplasms were adenocarcinomas with minimal variation in the architecture of individual growths (Fig. 10).

Gross metastases were not apparent. Transplantations were not attempted.

Changes in Other Tissues

General influence.—The generalized effect of the constantly absorbed estrogen was apparent immediately from the diminished size of the animals, but in the absence of complications or secondary infections their general health did not appear seriously impaired.

Genitalia.—Ovarian function remained in abeyance, the follicles persisting without evidence of further development or the formation of corpora lutea while

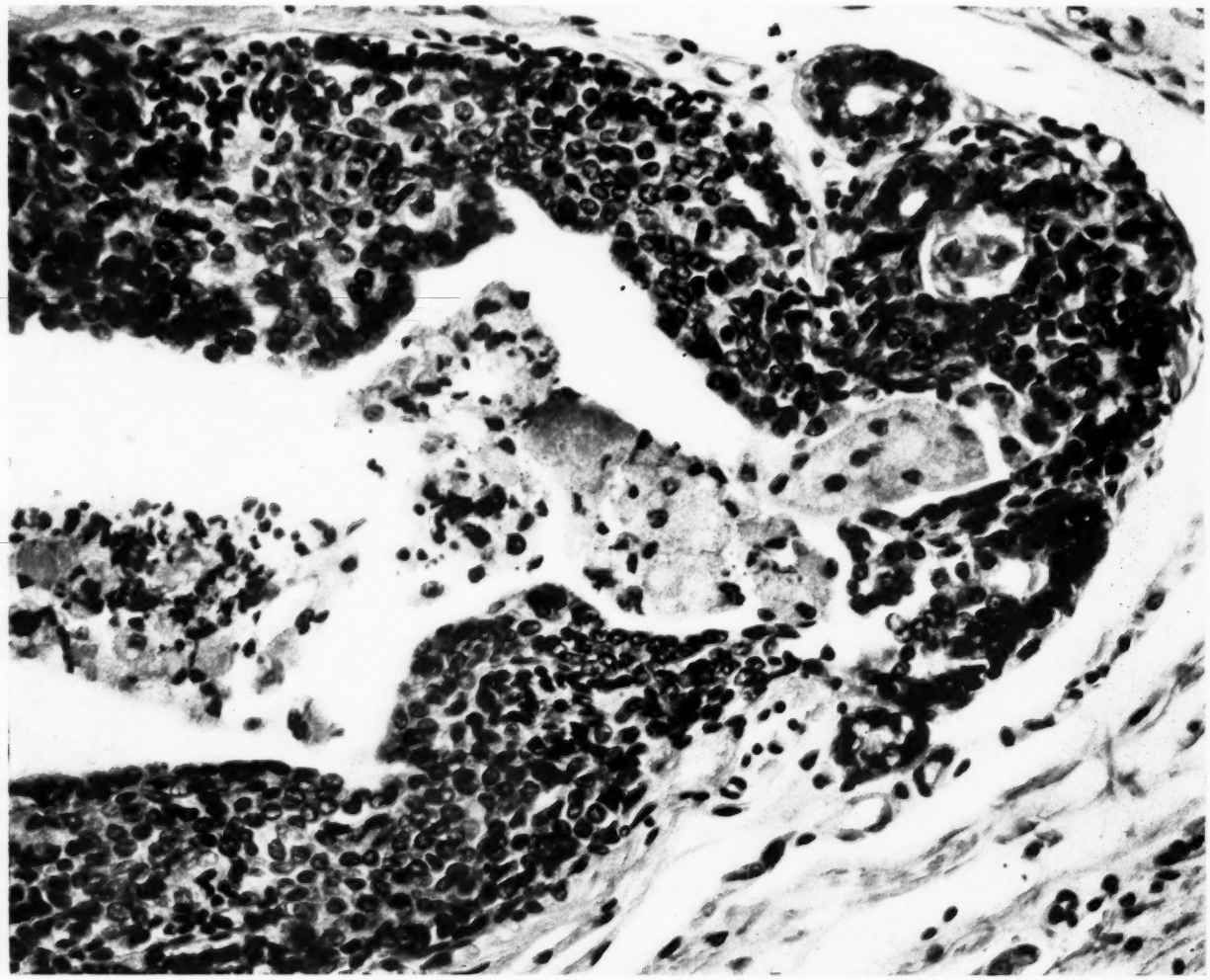


Fig. 7.—Male. Late secondary epithelial proliferation in an enlarged mammary duct situated in a fibrotic stroma. Note the secondary glands and the mitotic figures, but the essentially benign nature of the process. Estradiol dipropionate, 16.0 mgm., 404 days. Mag. × 375.

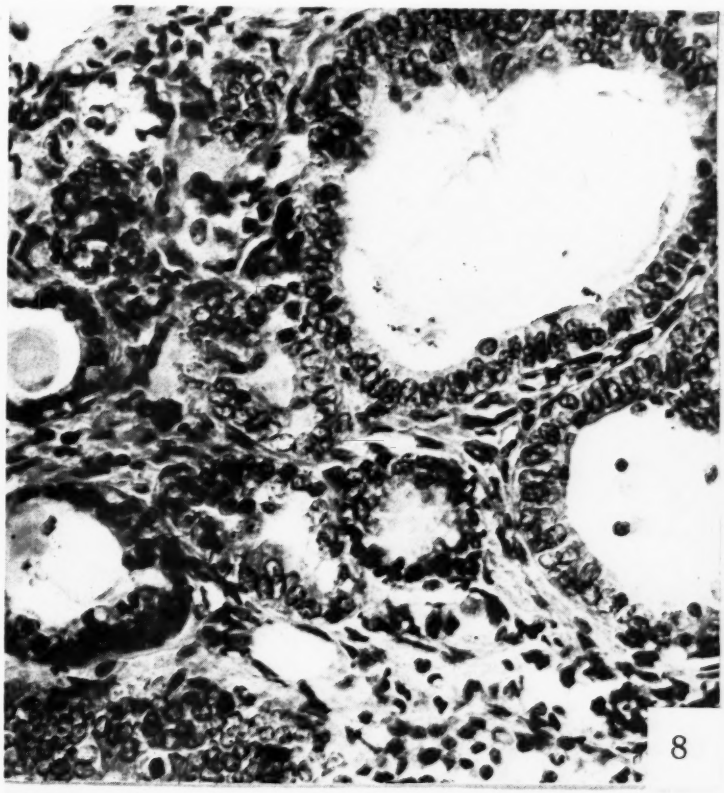
Description of Figures 8 to 11

Fig. 8.—Female. Multiple foci of late secondary epithelial proliferation in mammary ducts. Note the frequent mitoses, some atypical cells, but the absence of the disorder of cancer. Estradiol dipropionate, 11.5 mgm., 333 days. Mag. × 200.

Fig. 9.—Female. Mammary adenocarcinoma situated in an area of chronic fibrocystic disease. Estradiol dipropionate, 12.5 mgm., 376 days. Mag. × 200.

Fig. 10.—Female. Mammary adenocarcinoma. Estradiol dipropionate, 1.0 mgm., 803 days. Mag. × 375.

Fig. 11.—Squamous cell cancer of the cervix. This tumor developed in the same animal as the mammary lesion illustrated in Fig. 6. Mag. × 200.



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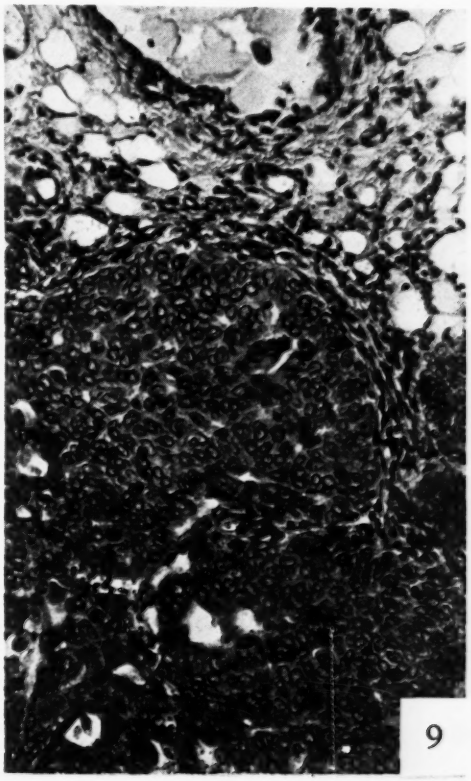

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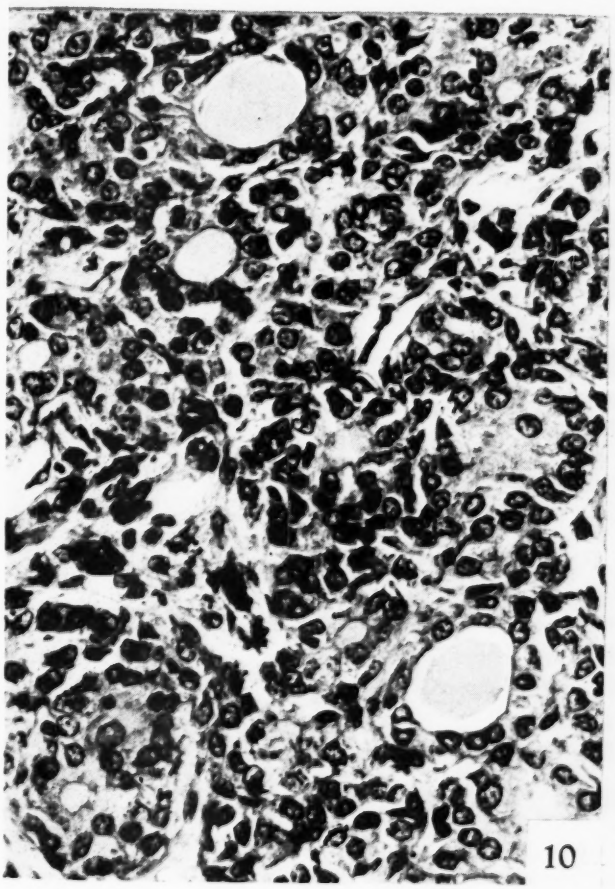

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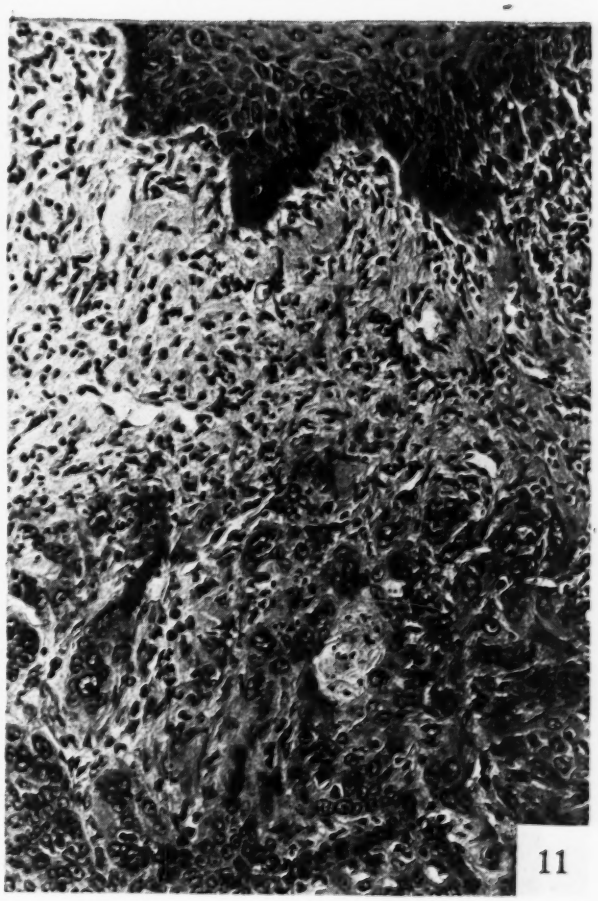

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FIGS. 8-11

implanted estrogen continued available. Cystic changes were common in animals surviving 1 year or longer.

Initially some signs of proliferation were manifest in the cylindrical epithelium of the uterine horns. Squamous cell metaplasia followed in many instances, while in a considerable number pyometra developed as a final complication. It was surprising that animals with such enormous but encapsulated masses of purulent material survived for many months. In the absence of infection, hyalinization developed, at first in the submucosa, later in the entire uterine wall.

In the cervix benign proliferation of the squamous cell zone was an invariable finding. One animal, killed after 551 days, had a squamous cell cancer of the cervix (Fig. 11). No vaginal tumors were found.

In the male the undescended, stunted testes remained functionally in a rudimentary state. The small seminiferous tubules were lined by 2 or 3 layers of cells which showed no sign of spermatogenesis. Evidently these alterations were reversible, for normal development eventually took place in estrogenized animals if, following the complete utilization of a supply of estrogen, the exogenous hormone was not replaced (13). In the seminal vesicles, flattening of the nonfunctioning epithelium accompanied by fibrosis of the wall of the organ appeared in all animals.

Adrenal gland.—Prominent changes were not found. Small cortical cysts appeared in some rats after 1 year.

Hypophysis.—Hyperplasia of the anterior lobe has been described consistently in estrogenized animals. In the present series it was evident that the time of appearance of this change was delayed as a result of slow absorption of the responsible hormone. Rats surviving 150 days or longer showed some hypophyseal enlargement, but the degree appeared unrelated to the lesions in other tissues.

Other organs.—Consistent evidence of change was not discernible in the thyroid, thymus, or pancreas.

Other Tumors

Lymphosarcoma.—Lymphosarcoma, originating in the mesenteric lymph nodes, was present in 2 rats, a male that received 1 mgm. of estrogen and died after 338 days and a female treated with 17.5 mgm. which succumbed after 359 days. As these growths have been observed in untreated animals of this strain their relationship to estrogen, if any, is difficult to evaluate.

DISCUSSION

From the results of these investigations it is readily apparent that slow constant absorption of an estrogen, estradiol dipropionate, from paraffin foci containing the crystalline material, produces in rats of known genetic constitution and of either sex a chronic proliferative mammary lesion which is preeminently benign. The induced alterations, which affect primarily the ducts, although continuous parts of a single basic process, pass imperceptibly and without uniformity in different animals or in various regions of the breast of one animal through the consecutive stages of proliferation, secretion, dilatation, and cystosis. These expressions of abnormal estrogenic activity, primarily affecting the epithelium, are succeeded by fibrotic changes in the supporting tissue which are initially periductal but which ultimately become more intense and finally involve large areas of the interepithelial tissues. Not without some justification the characteristic diffuse lesion found in many rats after 180 days or more may be considered an experimental counterpart of chronic cystic mastitis of the human subject.

The term "chronic cystic mastitis" is acknowledged by most investigators as unfortunate. Nevertheless, it continues to be employed to designate an ill defined group of benign mammary lesions although it is now universally recognized that inflammation plays no etiologic role. An endocrinologic basis for the condition suggests itself from many of its outstanding clinical aspects, such as: the occurrence in persons relatively young and still sexually active; the frequent and consistent variation in degree of the lesions, both objectively and symptomatically, with the menstrual cycle; the commonly associated menstrual disorders; the evidence of other abnormalities in the sexual apparatus or the history of earlier deviations from the normal; and the amelioration often produced by hormonal therapy. Although Taylor (44) finds no definite proof of an endocrine basis, he does admit that ovarian dysfunction cannot be entirely excluded in patients with the syndrome. In a more recent survey, containing an admirable account of earlier investigations, Taylor and Waltman (45) state that the long continued injection of mice with estrogen in oil, while eliciting adenomatous proliferation of the mamma in animals hereditarily susceptible to cancer and duct hyperplasia in other strains, fails to induce the fibrocystic lesion with complex epithelial proliferations that is so characteristic of human chronic mastitis. Geschickter (23), on the other hand, who bases his contention on urinary hormone assays, believes that a reduction in the elaboration of the corpus luteum hormone associated with hyperestrinism is responsible for the disease. In rats, according to this author, it can be reproduced by treatment with excess of estrogen combined with progesterone or testosterone. Grumbrecht (26), arguing from experimental evidence in the rat, unhesitatingly classifies chronic cystic mastitis as an endocrine abnormality.

In employing the rat with the object of simulating experimentally a human disorder as variable in its

manifestations and at the same time as extensive in scope as chronic cystic mastitis, it is important to realize the existence of biologic and physical differences in the normal mammary tissue of the species. While it is impossible to evaluate the significance of these differences, they must operate as factors conditioning the comparability of the spontaneous and induced forms of an endocrine disease. Taylor and Waltman (45) regard 2 alterations as characteristic of chronic mastitis: adenofibrosis and duct hyperplasia. Geschickter (23) prefers the terms mastodynia and adenosis for essentially the same manifestations. With justice the former state that, for an investigator to assert that the production of chronic mastitis has been achieved experimentally, "a fair proportion of the morphologic forms of the human disease must be demonstrable."

The late stages (Figs. 4 to 8) of the lesions in the mamma of the rats employed in the present experiment, produced by slow but constant absorption of estrogen, appear to imitate chronic cystic mastitis in the human subject closely enough to allow the supposition that one of the factors responsible for this disease is prolonged excessive or abnormal influence by the female sex hormone. The fibrosis is one of the cardinal late features of the experimental lesion, and to judge from a study of successive stages it is secondary to proliferation and function in the duct epithelium, and more especially to dilatation of the duct lumina by secretions. The subsequent distortions, contractions, and disorganization of persisting mammary ducts are physical complications occasioned by the stagnated secretions and this new abnormal mass of connective tissue, subject itself to hyalinization.

The late secondary epithelial proliferations, occurring in a few animals with fibrocystic mammary disease, form the logical counterpart of the duct proliferations that are such an important feature of the disorder in the human breast. In both species it is difficult to appraise the significance of this process. The cells appear unquestionably to be proliferating vigorously. However, the absence of genuinely atypical qualities confirms their essentially benign nature. Whether malignant change is the ultimate fate of cells of this type in subjects surviving for a sufficient time, is manifestly impossible to ascertain. The supposition appears tenable. These cells, rather than the remainder in an exhausted or resting stage, do give the appearance of renewed proliferation after the completion of a cycle of growth, function, and cessation of activity through the operation of a constant hormonal stimulus. The sequence is not unlike that observed by Greene (25) in certain types of spontaneous cystic mammary disease and cancers in the rabbit.

A question of paramount importance remains: the relationship of the female sex hormone to mammary cancer. Is there sufficient evidence for the assertion that the estrogens are capable of functioning as carcinogenic agents? In our present state of knowledge it is perhaps useless to probe too deeply into the concept "carcinogen." It is sufficient to recall the diversity of agents which may elicit neoplasia either occasionally or inevitably: physical factors such as ultraviolet light, radium, or roentgen radiations; many otherwise indifferent chemical substances which induce tumors only when present in abnormal concentration or at unusual sites, as sugars and acids; certain azo dyes which induce growths in the liver; cysticercus larvae, giving rise in the rat to sarcoma of the liver, the normal habitat of the parasite, but capable of causing tumors at other sites as well, as, for example, after they have been transported artificially to the subcutaneous tissue; and finally the carcinogenic hydrocarbons, the most potent of all, with a more generalized activity and surmised to act directly and locally on the cell to transform it irrevocably into a malignant subtype. The exact position of the estrogens in this series is debatable.

In the experiments recorded in this communication the late secondary epithelial proliferation, while essentially a benign manifestation of chronic fibrocystic mammary disease yet suggestive of possible subsequent malignant alteration, was observed in 6, and lesions interpreted as mammary cancer were found in 2 of 103 estrogenized rats that survived for 242 days, the minimal time at which these proliferative lesions were evident. Serial examination, instead of isolated sections from representative macroscopic zones, would perhaps have yielded a greater incidence, especially of the earlier type, but it is unlikely that the high incidence of cancer newly reported by Geschickter and Byrnes (24) in estrogenized rats would have been approached.

The incidence (8 per cent) of combined late proliferations and cancer of the breast in estrogenized rats, mentioned in the preceding paragraph, is not negligible, however, if compared to the virtual nonexistence of these lesions spontaneously in the strains employed. On this assumption—the almost certain likelihood that the lesions will not occur in the absence of the treatment instituted—abnormal estrogenic stimulation can be classified conditionally as one of the factors responsible for carcinoma of the breast. The relationship of the hormone to the multiple breast cancers first observed in one animal 803 days after the single administration of 1.0 mgm. crystalline material is hard to determine, for in an animal given this dose the exogenous estrogen must have disappeared some time prior to the inception of the tumor. The single instance of carcinoma of the cervix among

many examples of benign proliferations of this part in estrogenized rats, while not comparable with the incidence of cervical cancer observed by Allen and Gardner (2) in mice given estrogens, is of interest, since a comparable tumor in untreated rats of the strain is as yet unrecorded. Although carcinomas occurred in rats that had received estrogen, there are, nevertheless, serious objections to admitting that estrogen, even though acting under artificial conditions, functions directly as a carcinogen:—

1. Slowly and constantly absorbed exogenous estrogen acts only on those tissues normally under the control of a physiologic supply of the endogenous hormone. True carcinogens possess a more universal scale of potency.

2. Tumors, either sarcoma or mammary carcinoma, are not produced locally at the point of maximal concentration of the crystalline hormone incorporated in paraffin, a vehicle inducing a mild, nonspecific, localized irritative effect. It may be added, however, that a few sarcomas have been recorded by Gardner and his associates (19) in mice at the site of injection of estrogen in oil.

3. The generalized mammary alterations produced by estrogen pass through a complete cycle of cellular proliferation, function, and exhaustion despite the constant presence of the original inciting agent. The malignancy of the early proliferations, considered by Geschickter (22) to be cancer, is manifestly disproved by the subsequent course of these lesions. It may be logical to assume, especially with the analogy of the local action of carcinogenic hydrocarbons in mind, that the late proliferations, leading ultimately in some cases to true neoplasia, are related to some superimposed local phenomena the nature of which cannot be surmised.

The mode of action of the carcinogenic hydrocarbons is strikingly different from that of the estrogens. The changes progress from an indifferent or benign reaction to frank malignant disease, and if a sufficient concentration is available for an adequate period practically all the treated animals develop malignant tumors.

An additional hypothesis, not susceptible of proof, has been suggested for the origin of cancer in estrogenized mammary glands. The hormone increases the total amount of breast tissue and also, therefore, the probability of chance occurrence of the late alterations, an event which would be equally liable to occur if the increase were wholly constituted by normal elements.

The mammary tissue of female mice of strains susceptible to spontaneous mammary cancer presents some differences from that found in resistant mice. Gardner, Strong, and Smith (20); Fekete (16); Taylor and Waltman (45); and many years earlier, though not in inbred strains, Haaland (27), all record persisting foci of adenomatous hyperplasia in susceptible strains prior to the development of tumors. These probably constitute the precocious morphologic criteria of a hereditary predisposition to malignant disease, manifested in a localized area of specific tissue. The administration of estrogen to such animals accentuates this development. Lesions like this do not arise under estrogen in mice of resistant strains, which instead show only cystic changes in secreting ducts. Lacassagne (30) considers these two processes to be a manifestation of different inherited susceptibilities to the hormone.

The effects of estrogen on the rat, well known to be highly resistant to mammary cancer, include, in addition to the changes occurring in resistant mice, a tendency to secondary fibrosis of the supporting tissues. All this appears to indicate that the rat, on the whole, may logically be classified with the insusceptible group of mice.

SUMMARY

Crystalline estradiol dipropionate, implanted in rats of known genetic constitution and from 3 to 5 weeks of age, induced chronic diffuse alterations in the genitomammary system of both sexes, among which a fibrocystic mammary disorder, preeminently of a benign nature, was the outstanding manifestation. The lesions in the breast developed as a sequence of events characterized by considerable variability but occurring in all animals. The initial manifestations were present in the epithelium and consisted in proliferation, chiefly of the ducts; signs of functional activity by the cells; microscopic, followed by macroscopically visible enlargement of the lumina as a consequence of accumulated secretions; and exhaustion of cellular activity with subsequent evidence of disintegration of the cells or reversion of the epithelium to a resting stage. These lesions appeared responsible for the succeeding changes in the stroma. Proliferation of the fibrous tissue about cystic ducts was followed by diffuse fibrosis. This gave rise to contraction and distortion of the persisting mammary elements, and resulted ultimately in the production of a state comparable in many respects with human chronic cystic mastitis. With the later development of somewhat more atypical foci of secondary proliferation of the duct epithelium in the involved zones, this comparison appeared even more appropriate. Lesions interpreted as mammary adenocarcinoma occurred in 2 rats. Of associated changes in other tissues, a single example of squamous cell cancer of the cervix was of interest. The manifestations in animals that received estrogen have not been observed in untreated control rats.

While the etiologic agent which produces in the rat a chronic, benign, fibrocystic mammary lesion was estrogen, slowly but constantly absorbed from a focus containing the crystalline material, it is concluded that additional factors of an unknown nature, possibly acting locally, are more directly concerned with the origin of the late secondary epithelial proliferations and true neoplasia.

It is a pleasure to acknowledge the cooperation and advice of Dr. Francis Carter Wood.—AUTHOR.

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Tumor Inhibitor Studies

I. The Effect of Pure Chemical Compounds on Tumor Takes*

B. E. Kline, M.S.,** W. L. Wasley, Ph.D., and H. P. Rusch, M.D.

(From the McArdle Memorial Laboratory, the Medical School, University of Wisconsin, Madison, Wis.)

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Although a large number of chemicals have been tested for tumor-inhibiting qualities, the results usually have been discouraging. In general, positive findings have been limited in scope and application. Success in other fields of chemotherapy, however, lends encouragement and stimulation to further research in this field. As part of a program designed to investigate the influence of certain tissue extracts on cellular proliferation, we have had occasion to test a number of pure chemicals for similar effects.

PROCEDURE

Young albino Sprague-Dawley rats of both sexes weighing from 70 to 100 gm. were used. The tumor employed was the Flexner-Jobling carcinoma, which grows vigorously in from 90 to 95 per cent of these animals. The animals were kept on shavings in metal box cages, and Conkey's dog food and water were available at all times. The compounds to be tested were dissolved at twice the final concentration desired in 0.8 per cent NaCl solution, and the pH was adjusted to approximately 7.0. Each solution was tested for isotonicity to red blood cells by adding a drop of oxalated blood to a tube containing 0.5 cc. of solution and 0.5 cc. of saline. This concentration was the same as that to which the tumor cells were to be subjected. Those solutions which in 45 minutes caused hemolysis or severe crenation were diluted with isotonic saline until they did not affect red blood cells, and the lower level was used for testing. Relatively dilute solutions of the inhibitors could be used because the tumor cells were placed in direct contact with them.

The technic used in testing for possible inhibiting qualities of a compound was a modification of that described by MacFadyen and Murphy (7). Vigorously growing tumor was removed aseptically, cleaned of any extraneous or necrotic tissue, and forced through a small mincer having a plate 2 cm. in diameter with holes approximately 0.6 mm. in diameter. The mince was suspended in 2 volumes of 0.8 per cent NaCl solution, and 0.5 cc. of this was then thoroughly mixed with the same volume of a test solution by drawing back and forth in a syringe. As a control, the same procedure was used except that the tumor suspension was mixed with an equal volume of saline instead of the test solution. After these suspensions had stood for 30 minutes at room temperature, rats were inoculated subcutaneously with 0.2 cc. of the untreated (control) tumor in the right groin and with 0.2 cc. of a treated suspension in the left groin, using an 18 gauge needle. Movement of the syringe before each injection maintained the suspension and insured approximately the same number of cells in each inoculation. From 3 to 5 rats were used for each test, and all positive or questionable experiments were repeated. Tumor growth was followed for 2 or 3 weeks with measurements at weekly intervals.

In order to standardize the technic as much as possible, the following requirements were set up:

1. The test solution must be neutral and must not cause hemolysis or severe crenation of red blood cells after 45 minutes' contact.

2. The nuclei of hematoxylin and eosin stained preparations of tumor cells which have been in contact with the test solution for 45 minutes must appear normal histologically.

3. The control tumor must grow rapidly and continuously.

4. Tumor inhibition: (a) was classed as complete if there was no growth of the treated tumor; (b) was classed as partial if no tumor arising from treated material was more than one-half the size of the control tumor; (c) must be evident in every animal of a group.

5. The test animals must remain in good health during the test period. There must be no weight loss.

If there was any question whether inhibition was present, the experiment was repeated. If results did not conform to the criteria specified above, they were classed as negative. Thus, some compounds which gave mild or partial inhibition on some tests were recorded as inactive if the results were not reproducible.

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** Finney-Howell Research Fellow.

RESULTS AND DISCUSSION

This method of testing for tumor inhibition has advantages as well as limitations. It has the advantage of eliminating the problem of individual variation within a group, since each animal serves as its own control. It is therefore possible to use a relatively small number of animals for each test. It is perhaps the most delicate method of testing low concentrations of water-soluble chemicals and is also a technics but this is a general criticism of any test of tumor inhibition. In this experiment, tumor cells have not yet become established in the host, therefore inhibition of growth of the cells will influence the take of the implant. In this respect it is comparable to a study of the effect of chemicals on the growth of tumor cells in tissue culture, with subsequent inoculation of animals to determine whether the treated cells will proliferate. It should be pointed out that this is not

TABLE I: EFFECT OF SOME CHEMICALS ON TAKES AND GROWTH OF THE FLEXNER-JOBLING CARCINOMA

Compound	Concentration, mgm. per cc.	Inhibition
β-Indolebutyric acid	5.0	+
	2.5	+
	0.5	+
	0.05	—
β-Indolepropionic acid	10.0	+
	5.0	±
	2.5	±
	0.5	—
Tryptophane and β-Indolepropionic acid	5.0 + 10.0	+
Sodium trichloracetate	50.0	+
	25.0	±
	12.5	—
Chloral hydrate	5.0	+
	2.5	+
	1.25	+
	0.5	—
Chloretone	5.0	+
	2.5	—
Lysine *	5.0	—
Arginine	0.5	—
Proline	0.5	—
Hydroxyproline	0.5	—
Glycine	50.0	—
Tryptophane	5.0	—
Guanidine *	2.5	—
Creatine	0.5	—
Allantoin	2.5	—
Alloxan	0.5	—
Avidin	2.5	—
Malonic acid	0.5	—
Ammonium acetate	5.0	—
Traumatic acid	5.0	—
Parabanic acid	2.5	—
Dimethylparabanic acid	2.5	—
Creatinine	0.5	—
Neurine	5.0	—
Uracil	1.5	—
Histamine phosphate	0.25	—
Thiourea	0.5	—
Urea	2.5	—
Methyl urea	0.5	—
Ethylidene urea	3.0	—
2-Imidazolone	2.5	—
2-Imidazolone-4-carboxylic acid	2.5	—
Ethanolamine	5.0	—
Ethanolamine trichloracetate	5.0	—
Ethanolammonium glycinate	5.0	—
Sulfanilamide	0.5	—
Sulfaguanidine	2.5	—
p-Aminobenzoic acid	2.5	—
Pyridine-HCl	2.5	—
Pyridinesulfonic acid	2.5	—
Nicotinic acid	5.0	—
Nicotinic acid amide	5.0	—
Trigonelline	5.0	—
Calcium pantothenate	0.5	—
N-(α,γ-dihydroxy-β,β-dimethylbutyryl) taurine	2.5	—

We are indebted to Dr. E. H. Volwiler of the Abbott Laboratories for a generous supply of β-indolebutyric acid and for malonic acid.

* These compounds gave partial inhibition in some tests but the results could not be obtained in all animals in every test.

\+ = complete inhibition.

± = partial inhibition.

— = slight or no inhibition.

fairly rapid procedure. It has the disadvantage that the inhibiting qualities of compounds would be missed if they cause tumor inhibition by virtue of a systemic effect on the animal rather than by a direct action on the tumor cells. Also, any compounds which are toxic to normal and tumor cells alike might be classified as inhibitors by this method, although we have minimized this possibility by the use of low concentrations and by the other criteria listed above.

Information obtained in these experiments cannot be compared directly with experiments using other necessarily the same as treating tumors which are already established in the animals. The results have no direct practical application and should be regarded merely as quickly and easily obtainable aids in choosing compounds to be investigated by other methods.

Tests were made with 44 pure chemicals. The concentrations used and results obtained are listed in Table I. Most of the compounds had no effect on the growth of the tumor cells, but a few of them prevented the tumor from taking.

The indole compounds are better known for their

stimulatory effect on plant growth. However, Tanaka and Tuboi (8) reported slight inhibition of tumor growth with daily injections of potassium β-indole acetate. We obtained complete inhibition with β-indolepropionic acid at 10 mgm. per cc. and partial inhibition at 5 mgm. per cc. β-Indolebutyric acid gave complete inhibition with as little as 0.5 mgm. per cc. Additional indole derivatives are being tested. Fildes has shown (4) that indoleacrylic acid inhibited the growth of certain bacteria in the absence of tryptophane, but that even traces of tryptophane reversed the inhibition. We have found that tumor inhibition by β-indolepropionic acid is not a competition with tryptophane utilization, for adding 5 mgm. per cc. of tryptophane to the inoculum had no effect.

Sodium trichloracetate at relatively high concentrations (25 to 50 mgm. per cc. of tumor suspension) was responsible for complete inhibition with no histologically demonstrable injury to the cells and no toxicity to the host. This is an important observation because the acid is commonly used in making tissue extracts. Both the acid and the salt are difficult to remove completely from other substances which are soluble in water and alcohol and, therefore, might appear in the final preparation in considerable quantity. We found this substance responsible for the inhibition exhibited by a tissue extract which we had prepared using trichloracetic acid in one of the steps. The complete inhibition obtained with low concentrations of chloral hydrate and chloretone, which are closely related to trichloracetic acid, is very interesting. These compounds are hypnotics and their action on nerve centers may be the same as that on tumor cells. In this connection, Crabtree (3) demonstrated that certain chloro compounds, including monochloracetone, inhibited tumor glycolysis *in vitro* although treated cells grew if implanted into a rat. These results suggest that the inhibitor may have been quickly detoxified or eliminated by the animal. The observations with monochloracetone differ from the permanent inhibiting effects of the chloro compounds which are reported in this paper.

There is good evidence that sulfanilamide and sulfaguanidine inhibit the growth of certain bacteria, presumably by competing with *p*-aminobenzoic acid in some enzyme system (10). In view of the specificity of their action, it is of interest that these drugs had no effect on the tumor take. This might indicate that the biochemical system which is inactivated by the sulfa drugs in certain bacteria is absent or nonessential in the Flexner-Jobling carcinoma. On the other hand, the mechanism for the utilization of *p*-aminobenzoic acid in this tumor could easily differ from that found in bacteria.

Some of the compounds which were inactive in our tests have been reported by others to be tumor inhibitors. Complete tumor inhibition has been described by Lustig and Wachtel (6) with lysine and guanidine. In our hands, these compounds gave partial inhibition in several tests, but the results were not obtained in all animals in every test. Although these substances might be classed as weak inhibitors, they have been listed as negative according to our criteria. Lustig and Wachtel have also observed good inhibition with alloxan, which is known to inhibit enzymes that depend on $-SH$ groups for activity. Similarly, tumor inhibition by malonic acid, which inhibits the succinoxidase system, was described by Boyland (1), who also suggested activity by ethanolamine and other amines (2). It is difficult to compare our results with those referred to above because of differences in animals, tumors, and technics employed by the various investigators. Frequently authors have mentioned slight inhibition, which may not be significant since it could be caused by the toxicity of the compound for the host. Of course, temporary toxicity to the host is not objectionable if it causes complete and permanent regression of the tumor, but if the inhibition is only slight and temporary, the treatment is hardly to be recommended.

Avidin, the toxic protein of egg white, was tested to see if it would inactivate the biotin of the tumor and thus prevent growth. It was inactive, but we have no way of knowing that it actually permeated the cells, even though it is water-soluble. It is also possible that the cells contain an enzyme which can hydrolyze the avidin-biotin complex. Györgi and Rose (5) reported such an hydrolysis when they gave avidin-biotin complex to rats by intraperitoneal injection. They suggested that the complex is stable only in the digestive tract. West and Woglom (9) observed that a biotin deficiency produced by feeding avidin did not interfere with the takes or growth of 2 transplantable mouse tumors.

SUMMARY

More than 40 compounds were tested for inhibitory action on the Flexner-Jobling rat carcinoma by using a modified MacFadyen-Murphy technic.

Of the compounds tested, β-indolepropionic acid, β-indolebutyric acid, chloral hydrate, chloretone, and sodium trichloracetate prevented the tumor cells from taking.

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Heterologous Transplantation of a Human Fibrosarcoma*

Harry S. N. Greene, M.D.

(From The Rockefeller Institute for Medical Research, Princeton, N. J., and The Department of Pathology, Yale University School of Medicine, New Haven, Conn.)

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Several years ago it was found that rabbit cancers can be transferred readily to animals of alien species by utilizing the anterior chamber of the eye as an inoculation site (1). The ease with which such transfers were effected suggested that the same technic might be applied to the heterologous transplantation of human tumors. Accordingly, a series of transplantation experiments was undertaken and, at the present time, a considerable number of human sarcomas and carcinomas have been successfully transferred to guinea pigs and rabbits (2). In general, the major interest in such transfers has been centered on a study of the factors which permit the breakdown of species barriers and allow growth in the alien host, but in one instance attention has been directed particularly to the character of tumor growth in the foreign species.

The tumor selected for this study was a fibrosarcoma derived from the breast of a young woman.[1] It was transplanted 3 hours after operation on Mar. 7, 1940, and has now been carried by serial anterior chamber transfer in the guinea pig for more than 2 years. In addition, transplantation to the guinea pig's testicle has been successfully effected. The object of the present paper is to report the results of transplantation experiments and to describe the growth characteristics of the tumor.

MATERIALS AND METHODS

The primary transfer of the tumor has been described (2). Tumor fragments were transported from the operating room in a cooled thermos bottle and transplanted immediately upon arrival at the laboratory.

The technic of anterior chamber transfer employed throughout the experiments is extremely simple. After local anesthesia, the chamber is opened close to the upper border of the corneoscleral junction by a short, quick jab with a double-edged corneal knife. A small amount of fluid is allowed to escape, and a fragment of tumor tissue measuring less than a millimeter in diameter is inserted by means of a trocar. The corneal incision is not closed and, in order to avoid the possibility of escape, the fragment is forced into the inferior angle of the iris by applying pressure along the corneal surface with a blunt instrument.

Testicular transfer is performed in a similar manner. The skin and tunica vaginalis are incised with a corneal knife and a fragment of tumor inserted by means of the trocar used in anterior chamber operations. The incision is closed by a single suture or by the application of collodion.

The guinea pigs used were normal males and females varying from 3 months to 2 years of age. The majority were of a strain maintained at The Rockefeller Institute in Princeton, but others were bought in the open market. They were not subjected to special treatment either before or after transfer but were carried under ordinary conditions of cage life.

TABLE I: THE RESULTS OF TRANSPLANTATION OF A HUMAN FIBROSARCOMA INTO THE ANTERIOR CHAMBER OF THE EYES OF GUINEA PIGS

Generation number	Number of guinea pigs used	Number of takes	Takes, per cent
1	20	4	20
2	44	9	20.4
3	37	7	18.9
4	31	15	48.3
5	33	14	42.5
6	38	17	44.7
7	21	11	52.3
8	12	5	41.6
9	12	7	58.3
10	4	3	75
11	4	4	100
12	4	4	100
13	12	12	100
14	5	5	100
Total	277	117	42.2

RESULTS OF SERIAL TRANSFER IN THE ANTERIOR CHAMBER

The results of serial transfer in the anterior chamber of the guinea pig's eye are shown in Table I. The

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[1] This tumor was obtained through the cooperation of Dr. S. P. Reimann of the Lankenau Hospital, Philadelphia.

first transfer was undertaken on Mar. 7, 1940, and the last on Apr. 25, 1942. Takes were observed in only 4 of the 20 animals in the first instance but the percentage gradually increased and growth occurred in all the pigs of the last 4 generations.

The latent period between transfer and the appearance of signs of growth sometimes varied greatly in animals of the same generation but its average duration decreased consistently with continued passage. Growth was evident in 2 of the animals of the first generation on the 44th day, while in the remaining 2 the fragments remained unchanged for 130 days. In contrast, the latent period in the 3rd passage averaged 19 days and, in later transfers, growth could usually be detected by the end of the first week.

Occasionally signs of growth were delayed for very long periods of time. In several instances, tumor fragments remained unchanged in appearance for as long as 6 months yet subsequently underwent growth. Such transplants were attached to the iris but no vascularization was apparent on examination with a magnifying lens. They remained semitranslucent in contrast to the opaque white appearance characteristic of dead tissue, but showed no other signs indicative of continued life. Delayed growth of this type occurred in both young and old animals and no constitutional characteristic could be found consistently associated with the phenomenon. As a rule, animals were discarded if growth of the transplant was not apparent by the end of the 3rd month and it is probable, in view of the occurrence of delayed growth, that the number of takes in the various generations might have been increased with a longer observation period.

The first indication of growth in successful transplants was a pinkish coloration extending throughout the fragment and, on examination with a lens, this was found due to a diffuse invasion of the tissue by small blood vessels derived from the iris. Occasionally this state persisted for several months before actual size increase could be determined and in rare instances (1 per cent) terminated in regression rather than in growth. Usually, however, an immediate increase in size followed vascularization and proceeded uniformly and persistently in all diameters of the transplant.

The growth rate was relatively slow in early generations but increased with continued passage. Approximately 100 days were required to fill the chamber in the first generation, whereas in the last passage the chamber was completely filled in the majority of animals by the end of the 3rd week. In general, growth was more rapid in young animals but this was not a consistent finding and several instances of extremely rapid growth were observed in pigs more than 2 years of age. Growth was always greatly retarded during pregnancy but renewed increase followed parturition, and lactation appeared to be without influence.

The growing tumors were well supplied with blood vessels which were often sufficiently large to be seen traversing the surface with the naked eye (Figs. 1 and 2). Invasion of the iris occurred early and older transplants appeared as solid round masses protruding through the iris with an equal bulk in the posterior chamber. This appearance contrasts with that of rabbit tumors in alien species where growth occurs along the surface of the iris and fills the anterior chamber before invasion and posterior extension.

As a rule, the animals were killed when the anterior chamber became filled in order to utilize the tissue for other studies. In several instances, however, animals were held to determine the eventual fate of the tumor, and in the majority the sequence of events was the same. After filling the chamber, the tumor underwent partial regression. A small fragment of living tissue remained, however, and this in turn grew to fill the chamber again. Such a sequence was repeated in several cases and apparently the process may go on indefinitely. Fig. 5 shows a section of a transplant from an animal killed 332 days after transfer in which the process was repeated 3 times. Occasionally primary regression was complete while in other instances complete regression followed recurrent growth; but in the great majority of cases successive phases of partial regression and renewed growth continued throughout the period of observation.

Histologically the cells and structure of the transplants were identical with those of the primary human tumor (Figs. 3, 4, and 5). It should be noted, how-

DESCRIPTION OF FIGURES 1 TO 5

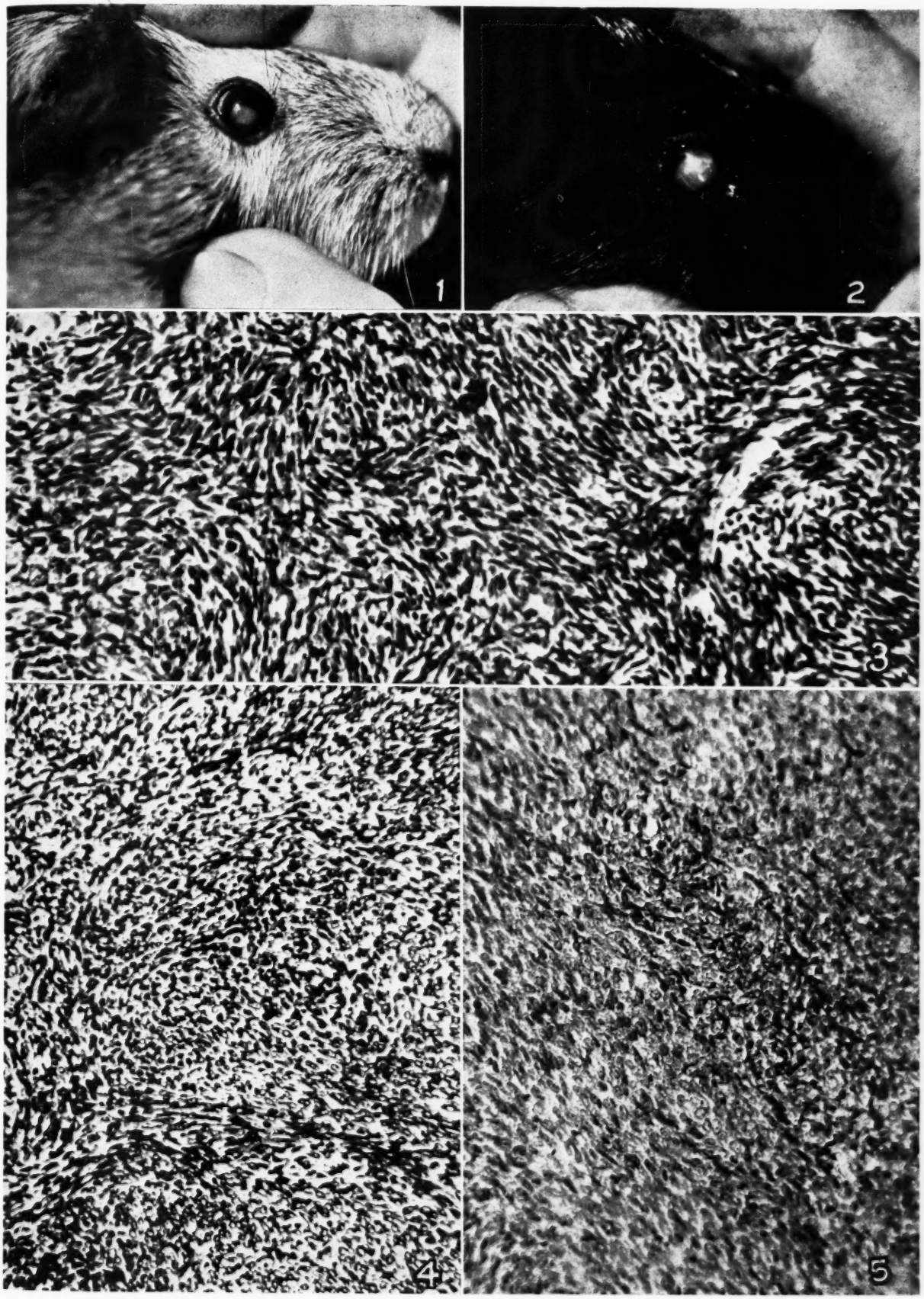
FIG. 1.—Eye of a first generation guinea pig containing a growing transplant of the human fibrosarcoma. Photograph was taken 187 days after transfer when the growth filled approximately one-half of the chamber. Actual size.

FIG. 2.—Eye of a 10th generation guinea pig 1 month after transfer. The growth almost entirely filled the anterior chamber. Actual size.

FIG. 3.—Section of the human fibrosarcoma used for transfer. Frozen section. Hematoxylin and eosin. Mag. × 225.

FIG. 4.—Section of a transplant of the tumor from the eye of a first generation guinea pig. The human tumor and guinea pig transplant are morphologically identical. Hematoxylin and eosin. Mag. × 225.

FIG. 5.—Section of a transplant of the tumor from the eye of a 3rd generation pig killed 332 days after transfer. An area of degeneration is present in the center of the photograph but the great majority of cells are intact and living. Hematoxylin and eosin. Mag. × 250.



FIGS. 1–5

ever, that in several of the more recent transplants there has been a tendency toward an adenoid arrangement of cells in the central portion with preservation of the typical fibrosarcomatous pattern elsewhere (Fig. 6). An architectural arrangement of this type was not found in many sections of the primary growth and the significance of the change is not clear. It is possible, despite histological appearances, that neoplastic epithelial elements were present in the original fragments used for transfer but it seems highly unlikely either that such cells would remain dormant for 2 years or that, by some fortuitous circumstance, they would be included in the 12 transfers undertaken during that period. Growths arising from transfer of fragments showing an adenoid tendency may or may not reproduce this pattern—the majority show a purely sarcomatous architecture. Further investigations are in progress.

Sections of early transplants show invasion of the iris together with an immigration of pigmented cells from the iris into the tumor tissue. Thin walled blood vessels are present in abundance and apparently arise from the margin of the iris about the periphery of the graft (Fig. 7). At a later stage the iris is completely replaced and pigmented cells are no longer found. Invasion of the iris, however, is limited to the area occupied by the mass of the tumor and there is no tendency to spread peripherally over the surface of the remaining iris or to infiltrate this structure laterally at points of contact.

In old or extensive growths a considerable portion of the posterior chamber may be occupied, with distortion of the lens and invasion and destruction of the ciliary body and retina. The sclera, on the other hand, remains clear and in no instance has invasion of this tissue been noted. In contrast, the cornea is occasionally involved but invasion is never pronounced and rupture with protrusion of the tumor is brought about largely by pressure atrophy. With continued growth, the ruptured tumor destroys and replaces the greater part of the eye, but the remaining portion remains freely movable in its socket and extension to the lid or other periorbital structure has not been observed. Old growths of this type may show extensive areas of degeneration and necrosis, but even in tumors judged from clinical observation to have completely regressed groups of intact and apparently living cells are usually found.

Lymphatic extension and metastasis have not been observed. Frequently circumscribed areas containing elements resembling the essential cells of the tumor have been found in the lung, but in view of the confusing and somewhat comparable histological picture incident to chronic inflammatory reactions in the guinea pig's lung, these could not be unequivocally identified as metastases. It should be noted in this connection that homologous tumor transplants in the anterior chamber of the eye rarely metastasize in less than 18 months, and no pigs bearing human transplants have been held under observation for this length of time.

Results of Testicular Transfer

Testicular transfers were successfully effected with tumor tissue derived from the 4th and 6th anterior chamber generations. The percentage of takes, however, was much lower than was obtained in the anterior chamber series. Two guinea pigs were used in the first experiment and one take resulted, while the 2nd transfer, with material from the 6th eye generation, gave rise to only 2 takes among 14 animals. Serial transplantation was attempted in the latter instance and transfer to 4 pigs resulted in a single growth. Thus a total of 20 attempts to transfer to the testicle gave rise to 3 takes, or a 15 per cent incidence of successful transplantation.

It is of interest, however, that if testicular transfer is successful growth is established earlier and proceeds more rapidly than in the anterior chamber. Palpable nodules were present in the testicles of all 3 animals 10 days after transfer, and in one the testicle was completely replaced by tumor by the 28th day. Growth was slower in the other animals and at autopsy, 58 and 61 days after transfer, it was found that the tumors occupied approximately two-thirds of the testicular parenchyma.

At autopsy the tumors were found limited to the testicle and there was no extension to the tunica

DESCRIPTION OF FIGURES 6 TO 10

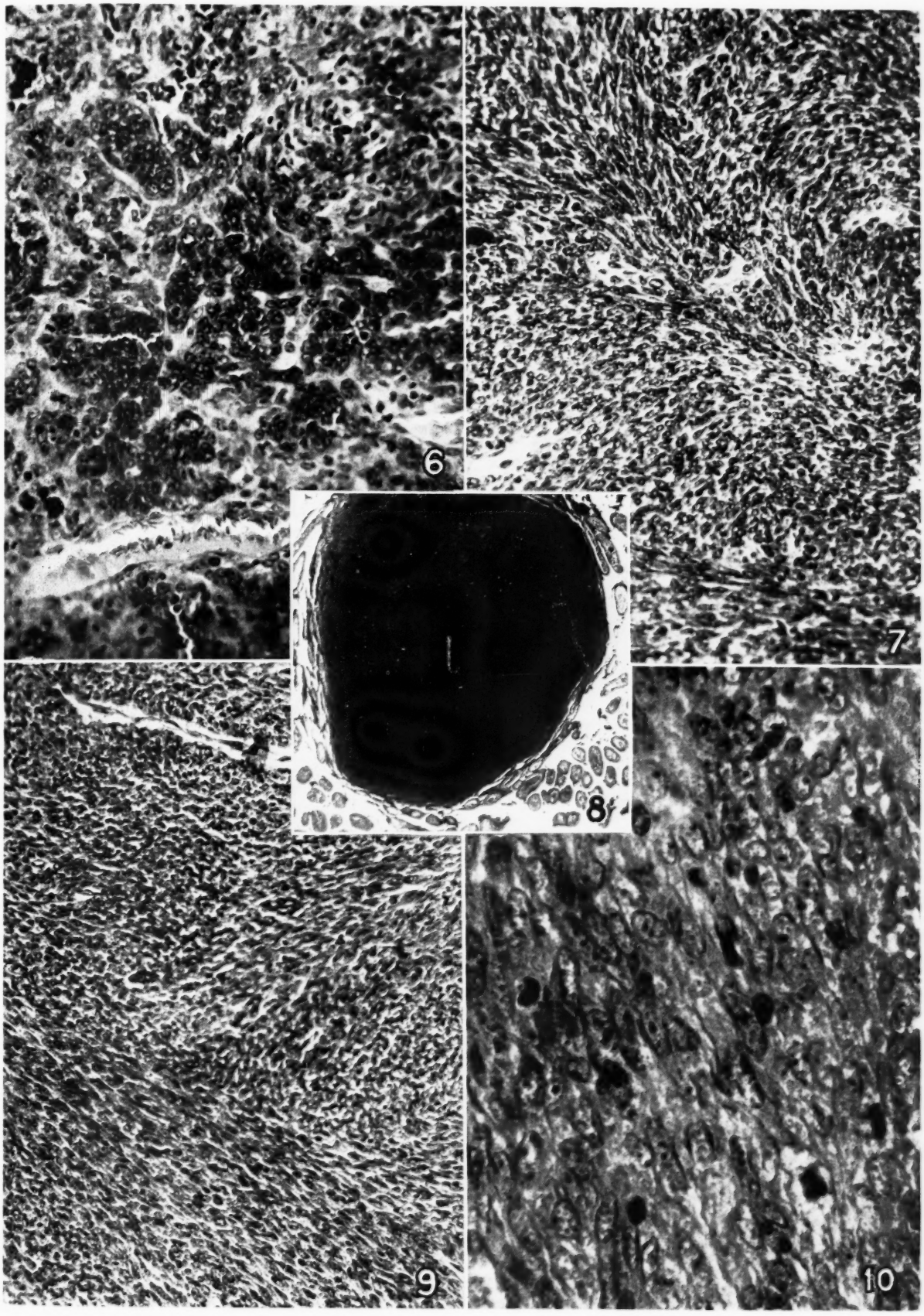
Fig. 6.—Section of the tumor in the eye of a 12th generation pig killed 42 days after transfer. The adenoid arrangement of cells occurred in the center of the transplant and elsewhere the typical fibrosarcomatous pattern persisted. Hematoxylin and eosin. Mag. × 225.

Fig. 7.—Section of the tumor in the eye of a 3rd generation pig killed 56 days after transfer. Numerous thin walled blood vessels are visible. Hematoxylin and eosin. Mag. × 225.

Fig. 8.—Section of a growing testicular transplant. Hematoxylin and eosin. Mag. × 13.

Fig. 9.—Higher power view of the transplant shown in Fig. 8. It is histologically identical with transplants in the anterior chamber of the eye. Hematoxylin and eosin. Mag. × 225.

Fig. 10.—Section of the testicular transplant from a 2nd generation pig to show more cellular detail. Hematoxylin and eosin. Mag. × 650.



FIGS. 6–10

vaginalis or into the spermatic cord. Histologically, the transplants were identical with the primary human tumor both in cellular constituents and in structure and there was an abundant vascular supply (Figs. 8, 9, and 10).

DISCUSSION

The point to be emphasized in the present connection is that human cancer can be transplanted to lower animal species and thus brought within the range of experimental investigation. The growths described in this paper fulfill all the requisites of successful heterologous transplants. They can be transferred serially and apparently maintained indefinitely by continued passage. Growth is progressive and, in several instances, has continued for more than a year with extensive invasion of orbital tissues. Metastasis has not occurred, but this is apparently a function of time for the ability of the tumor to grow in other parts of the body has been demonstrated.

The suggestion has been made that transplants of heterologous tumors in the anterior chamber of the eye are merely tissue cultures. This, emphatically, is not the case. The growths are supplied with stroma and blood vessels by the alien host and, in actuality, become part of this host. Furthermore, the tumors can be transplanted to the testicle without modification of the salient characteristics observed in the anterior chamber and in this locus their behavior is comparable in all respects with that of homologous testicular transplants of other tumors.

The ability of heterologous transplants to survive and to grow progressively in the testicle also demonstrates the fallacy of the argument that growth in the anterior chamber depends on the existence of a hypothetical barrier between the blood and aqueous humor which prevents the passage of antibodies into the chamber. In view of the success of heterologous testicular transplants, the maintenance of such an explanation would necessitate the construction of a similar barrier between the testicle and the blood stream and this is obviously absurd.

It would appear, on the other hand, that the ability to grow in an alien host, whether in the anterior chamber or in the testicle, is related to the nature of the tissue used rather than to a peculiarity of the inoculation site. Any living tissue might be expected to grow in an alien host—if growth was determined solely by the absence of inhibiting humoral reactions—yet neither normal adult tissue nor so called benign tumors, both of which are endowed with growth potentialities, possess the ability to survive in the foreign environment. It seems significant that this ability is limited to autonomous tumor tissue (3) and, as will be reported in a later paper, to normal embryonic tissue.

SUMMARY

A human fibrosarcoma was successfully transpianted to the anterior chamber of the eyes of guinea pigs and has been carried by serial transfer through 14 generations over a period of 2 years. In addition the tumor has been successfully transferred to the guinea pig's testicle.

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Mitochondria in Lymphocytes of Normal and Leukemic Mice*

J. S. Potter, Ph.D., and E. N. Ward, M.A.

(From the Carnegie Institution of Washington, Department of Genetics, Cold Spring Harbor, N. Y.)

(Received for publication May 1, 1942)

Studies on mitochondria in cases of chronic and acute lymphatic leukemia in man are not entirely in accord. In general, however, the reports indicate that the cells involved contain an increased number of very fine mitochondria in contrast to the moderately coarse structures found in normal lymphocytes (6). Lawrence and Todd (3) described 3 cases of lymphatic leukemia which were exceptions, in that 10 to 25 moderately coarse mitochondria were found in the mononuclear cells. These observations on number have been based for the most part on rough estimations, not actual counts. Wiseman (9), in attempting to correlate the number of mitochondria with the age of the lymphocyte, stated that "it is impossible to obtain accurate estimations of mitochondrial content, partly because of the physical difficulties involved in counting these bodies and partly because of the fact that some of the mitochondria occasionally fail to take the dye, while some fading of the stain occurs." Although Wiseman's paper is the only one dealing with the lymphocyte in particular, many others have shown that large numbers of mitochondria are present in young blood cells (2, 5).

As part of a comparative cytological study, this paper will present observations on the number, size, shape, and distribution of mitochondria in different populations of leukemic and normal lymphocytes in strain C58 mice.

Normal lymphocytes are defined as those found in the peripheral blood of mice showing no pathological condition. The data on normal cells were obtained from both young (average age 41 days) and older (average age 266 days) animals. The invaded peripheral blood was studied in cases of spontaneous leukemia (average age 479 days), and in 4 different lines of transplantable leukemias at the time of maximum invasion (hosts 4 to 6 weeks old). In the latter group, lines I and MLiv were both highly virulent with a survival time of 4 days, while lines S and U were less virulent, with a survival time of 7 to 15 days during the period of study.

Although fixed and stained material was also examined, the cells were studied for the most part in supravital preparations containing Janus green B and neutral red. Slides were prepared according to the following method after attempts had been made to find the most satisfactory combination for mouse blood: 9 drops of 1 per cent neutral red in absolute alcohol and 18 drops of 1 per cent Janus green (National Aniline, certified) in absolute alcohol were added to 10 cc. of absolute alcohol. Each slide was flooded with this mixture, drained, and allowed to dry dust-free. The blood films were made by touching a cover slip to a drop of blood and placing it on a prepared slide. Observations were begun immediately and counts made as soon as the dye had penetrated the cells. When a slide showed signs of cell death it was discarded and a fresh one prepared. For certain purposes dark field illumination was used, employing a Zeiss cardioid condenser and a 60 ×, 1.05 mm. iris oil immersion objective.

The mitochondria were counted in a random sample of 100 cells from each of 10 animals in the various classifications, giving a minimum of 1,000 cells examined for each type of blood. In most cases the authors made independent observations on the same supravital preparation.

OBSERVATIONS

Number of Mitochondria

By counting the mitochondria in random samples of populations of lymphocytes it becomes possible to present numerical values for the 7 classifications chosen. Table I gives a summary of ranges, means, and standard deviations of the counts made and comparisons among the 7 groups. It is shown that the differences are statistically significant, except between the 2 highly virulent leukemic lines MLiv and I, and between the less virulent lines S and U. The differences among the transplantable leukemias are otherwise statistically significant. There was some individual variation which may have been due to the method of random sampling. However, the number of mitochondria for 100 cells when grouped in classes of 5 gave good distribution curves. For each indi-

*This investigation was aided by a grant for the study of leukemia from the Carnegie Corporation.

vidual classification, the mean of each sample of 100 usually occurred in the same class. The distribution curves for the number of mitochondria in 1,000 cells from 4 classifications are presented in Fig. 1.

There is evidence that the number of mitochondria is correlated with the average cell size of a given population, the normal population having the smallest average size, spontaneous leukemia next largest, and transmissible leukemias the largest. Individual observations within each sample indicated, however, that large cells sometimes had small numbers of mitochondria and small cells at times contained large numbers. Direct evidence that the largest cells did not always have the most mitochondria is found in comparing the 2 transmissible leukemias, lines U and I. The mean number of mitochondria for line U was 34.42 ± 0.31, and that of line I, 32.07 ± 0.27, yet the mean diameter of cells in the line I population was 14.4 microns (volume 1563.5 cu. microns), while the mean diameter for line U was 12.8 microns (volume 1092.9 cu. microns).

Table I: Mean Number of Mitochondria in Populations of Lymphocytes, and Comparisons among the Different Groups Studied

	Number of cells	Range	Mean	Standard deviation
Normal (Y)	1,000	5–42	18.77 ± 0.20	6.40 ± 0.14
Normal (O)	1,000	4–48	21.57 ± 0.21	6.95 ± 0.15
Spont	1,000	8–56	27.93 ± 0.26	8.40 ± 0.18
Line I	1,000	12–60	32.07 ± 0.27	8.55 ± 0.19
Line MLiv	1,000	8–65	32.47 ± 0.28	8.95 ± 0.20
Line S	1,400	8–71	34.59 ± 0.29	11.00 ± 0.20
Line U	1,000	6–67	34.42 ± 0.31	10.04 ± 0.22

	Difference	Difference / S.E. of difference
Normal (O)-Normal (Y)	2.80 ± 0.29	9.6
Spont-Normal (Y)	9.16 ± 0.32	28.6
Spont-Normal (O)	6.36 ± 0.33	19.2
I-Normal (Y)	13.30 ± 0.33	40.3
I-Normal (O)	10.50 ± 0.34	30.8
MLiv-Normal (Y)	13.70 ± 0.34	40.2
MLiv-Normal (O)	10.90 ± 0.35	31.1
S-Normal (Y)	15.82 ± 0.35	45.2
S-Normal (O)	13.02 ± 0.35	37.2
U-Normal (Y)	15.65 ± 0.36	43.4
U-Normal (O)	12.85 ± 0.37	34.7
I-Spont	4.14 ± 0.37	11.1
MLiv-Spont	4.54 ± 0.38	11.9
S-Spont	6.66 ± 0.38	17.5
U-Spont	6.49 ± 0.40	16.2
MLiv-I	0.40 ± 0.38	—
S-I	2.52 ± 0.39	6.4
U-I	2.35 ± 0.41	5.7
S-MLiv	2.12 ± 0.40	5.3
U-MLiv	1.95 ± 0.41	4.7
S-U	0.17 ± 0.42	—

Perhaps the most significant correlation is between the age of the lymphocytes and the amount of mitochondrial material, a relationship already pointed out (9) for rabbit lymphocytes. In the present study and in earlier investigations (4) there seems little doubt that the larger cells in active lymphopoietic tissues of the mouse are the younger cells, judged by their ability to divide and the degree of basophilia. The proportion of immature cells and the rate of metabolism (7, 8) follows the same sequence in the 3 major classifications as did size; that is, normals lowest, spontaneous leukemias next, and transplantable leukemias highest. Within a virulent line of transplantable leukemia, samples taken daily during the progressive invasion of the blood by large, immature, and virulent cells showed an increase in the mean number of mitochondria (Fig. 2). A similar increase may be found in individual spontaneous cases with approaching death; in 3 cases where 2 counts were made on the same animal on different days, the mean of the 2nd count (3 to 9 days after the first) was the higher.

A sample of lymphocytes from a preparation of a normal lymph node teased in a drop of 0.9 per cent saline gave mitochondrial counts with a mean of 19.97 ± 0.53 and a standard deviation of 5.34 ± 0.37. This is not significantly different from the counts obtained on normal cells in peripheral blood. The mitochondrial content of a population of mononuclear cells from an active germinal center should be more nearly like that of a leukemic population, but sampling of that sort is at present technically impossible. Indirect evidence for this is the observation that immature normal cells contain a large number of mitochondria.

Size and Shape of Mitochondria

Certain variations in size and shape of mitochondria were seen within individual cells and among cells of a different population. A summary of the observations for each classification follows:

Normal.—Small discrete granules for the most part, although some cells contained rather indistinct granules; scattered rods and filaments.

Spont.—Both small and moderately large granules, discrete for the most part, but indistinct fuzzy granules were observed; scattered rods and filaments; a few ring-shaped forms.

Line I.—Large granules of swollen appearance also large and small discrete granules; scattered rods and filaments; rings rarely seen.

Line MLiv.—Large discrete granules for the most part; some small discrete granules; scattered rods and filaments; rings observed in 3 cases.

Line S.—A change occurred in the mitochondria of this line during the period of study. In transfers 85 to 90 the mitochondria were very small indistinct granules for the most part, although in some cases both moderately large and small granules and scattered rods and filaments appeared. In transfers 97 and 98 moderately large and small discrete granules and many long and short rods were seen.

Line U.—Great variability was observed in this line. In transfers 1 and 2 the mitochondria were very small indistinct granules. In transfer 3 large discrete granules, scattered rods, filaments, and rings appeared. In transfers 4 and 5 there were mostly large and small discrete granules and very few filaments or rods. In transfers 8 and 9 the mitochondria were small granules (not as discrete as in 4 and 5); a few rods were also seen.

Although in general the mitochondria in the lymphocytes of the mouse are small discrete granules, some rods and filaments were observed in each sample of the various populations. In all but lines MLiv and I a part of the mitochondria in each population was described as small and fuzzy or indistinct in outline. In the 2 highly virulent leukemias the mitochondria were consistently large sharply stained bodies which could be readily counted and photographed. This larger size seems to correct for the slightly higher mean number of the other 2 transplantable leukemias, and seems to permit a statement that, if not the actual number of granules, the total amount of mitochondrial material is correlated with size and physiologic age of the cell population. Fig. 3 reproduces photographs of typical normal cells, cells of spontaneous leukemia, and of lines I, MLiv, S, and U. The larger size of mitochondria in lines MLiv and I is apparent.

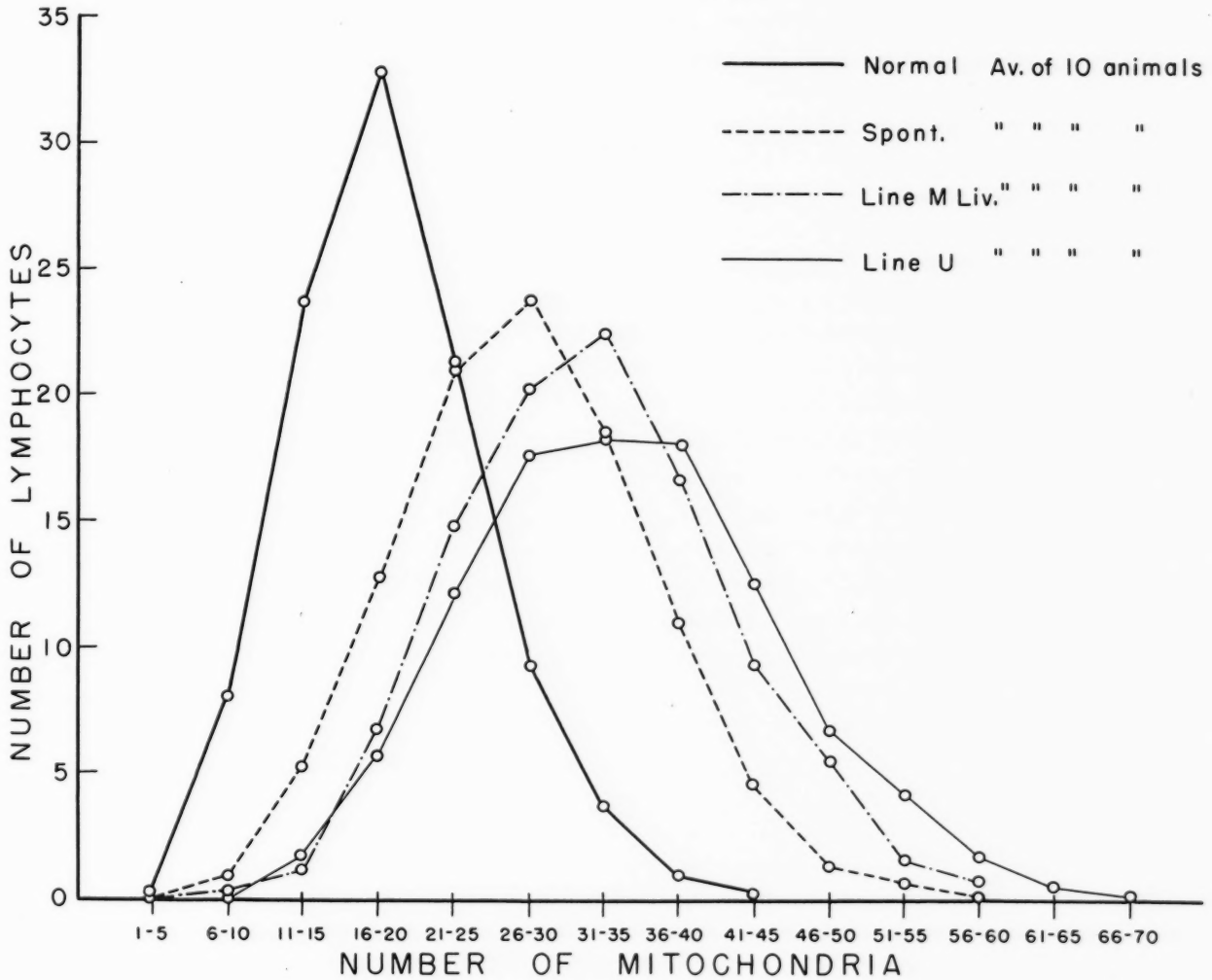

FIG. 1.—Distribution curves of numbers of mitochondria per lymphocyte for normal and for 3 leukemic cell populations.

Individual cells with large mitochondria were seen in the leukemic lines and in normal populations, but

large "swollen" mitochondria were noted particularly in line I transplantable leukemia. Since it has been reported that the enlargement of mitochondria sometimes accompanies cell injury (1), observations were made in both the supravital and dark field preparations to see whether any enlargement took place during the period of examination. Unless there was a change in the few seconds between removal from the mouse and the beginning of observation, it seems certain that no changes occurred. Neither did we find that there was any fading of Janus green during the time allotted for the examination of a supravital preparation. In unstained blood examined in a dark field, the larger size of the mitochondria in cells of transplantable leukemia MLiv as compared with line S was confirmed. It was therefore concluded that the consistently large mitochondria in the virulent leukemias were characteristic of the predominating immature cell type and not artifacts.

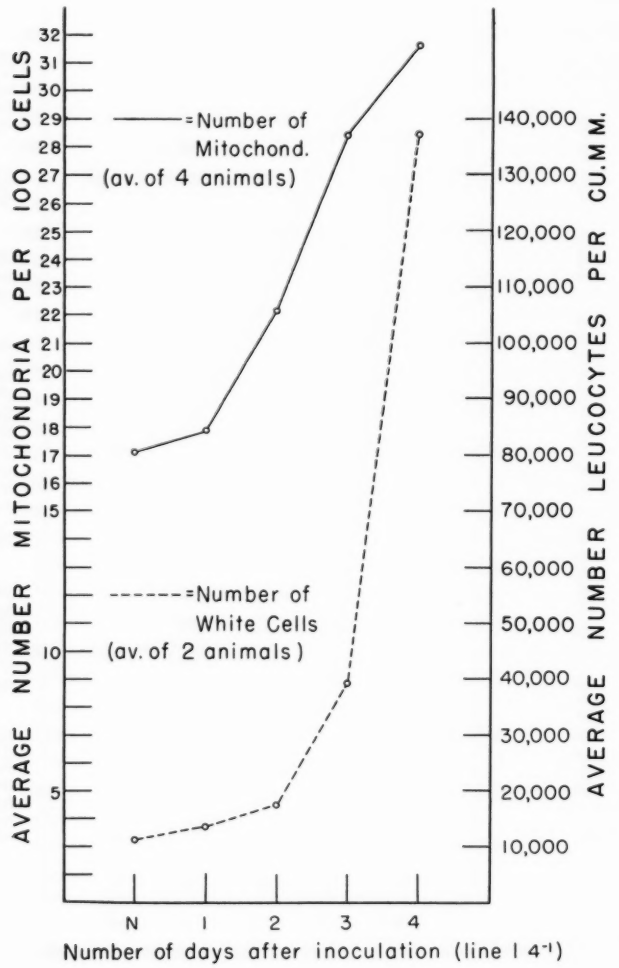
AVERAGE NUMBER MITOCHONDRIA PER 100 CELLS
AVERAGE NUMBER LEUCOCYTES PER CU.M.M.
——=Number of Mitochond. (av. of 4 animals)
------=Number of White Cells (av. of 2 animals)
Number of days after inoculation (line I 4-1)

FIG. 2.—Graph from daily series counts on line I leukemia showing parallel increase in average number of mitochondria per lymphocyte with increase in immature cells (leucocytes per cu. mm.) in peripheral blood.

DISTRIBUTION OF MITOCHONDRIA

The mitochondria in normal and leukemic cells apparently were not arranged in the cytoplasm or about the nucleus in a definite pattern. In focussing at different levels of a cell, mitochondria were usually found in each optical plane. Clumping of the mitochondria sometimes occurred but was not a usual pattern, nor was there any constant relation to nuclear indentation or to the nuclear membrane. Except during ameboid motion of a cell no movement of mitochondria in the cytoplasm occurred at an observable rate.

SUMMARY

Differences have been demonstrated in the average number of mitochondria among populations of lymphocytes from the peripheral blood of normal mice and mice with spontaneous or transplantable leukemia. Table I and Figs. 1 and 2 are presented to show differences in number, while differences in size are shown photographically in Fig. 3.

It is concluded that these population differences indicate different proportions of cell types, and that particular stages of differentiation of the normal and leukemic lymphocyte have the same mitochondrial characteristics. Therefore the pathologic cell is not characterized by aberrant mitochondrial content.

The data presented here agree with Wiseman's statement concerning rabbit lymphocytes (9), that the amount of mitochondrial material is indicative of the age of the cell, large basophilic cells having the greatest amount.

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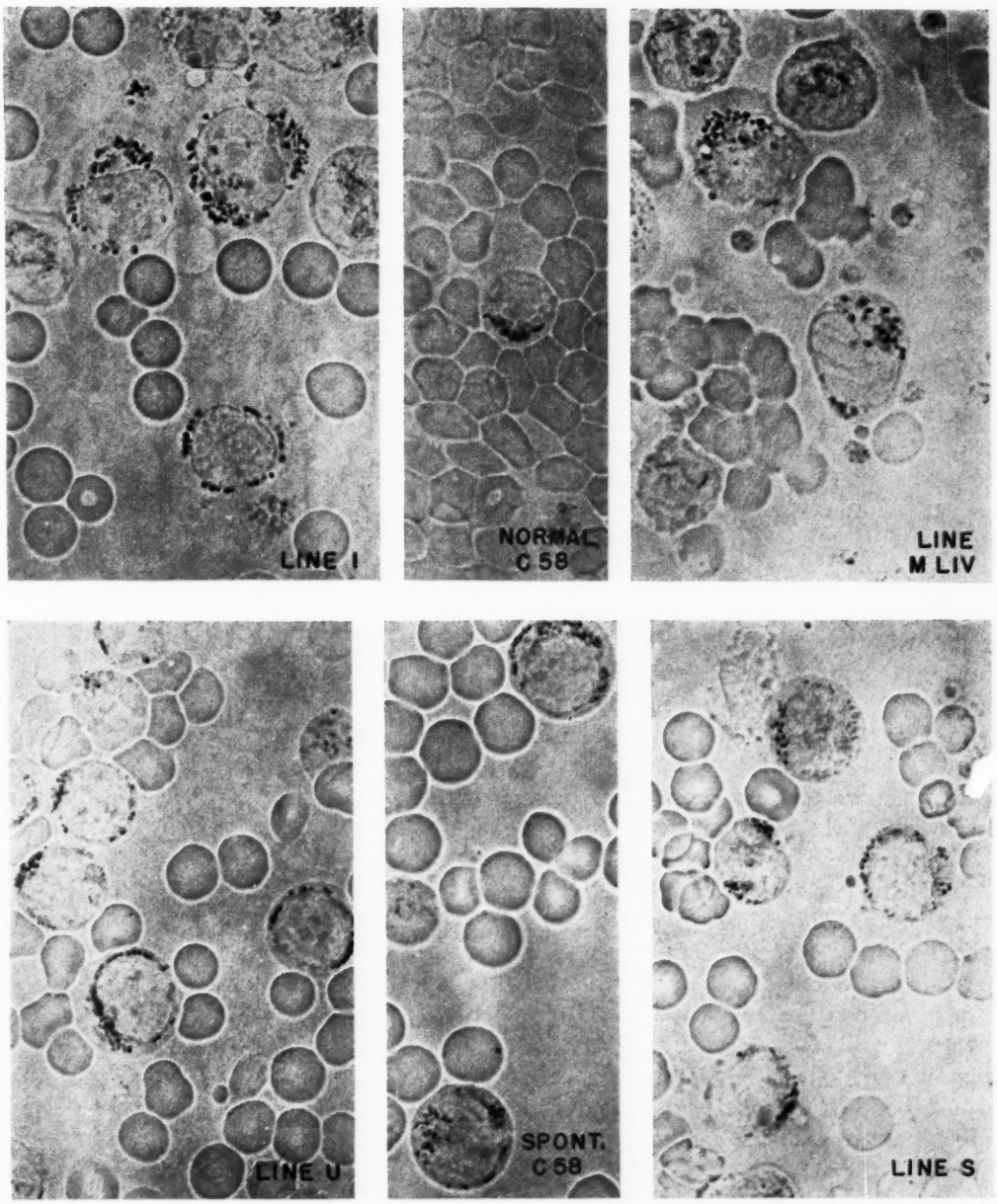

FIG. 3.—Photomicrograph of supravital preparation (Janus green B and neutral red) showing the mitochondria in lymphocytes of normal and leukemic mouse blood. Note larger size of granules in cells of lines MLiv and I. Mag. × 1000.

On the Mechanism of Tumor Production by Chemical Agents

Felix Bergmann

(From the Daniel Sieff Research Institute, Rehovoth, Palestine)

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One of the most promising lines of cancer research is a study of the relationship between the structure and activity of carcinogenic compounds, for it is possible to vary arbitrarily the former and to determine the influence of modification on the latter. However, in spite of the enormous amount of material that has accumulated, no clear general theory of this particular aspect of the cancer problem has been developed.

It was observed by Haddow (9, 10) that carcinogens inhibit the growth of young animals in a specific manner. He suggested that as a result of this general growth inhibition certain cells may be so fundamentally changed as to acquire a new hereditary character and to be no longer subject to the regulating influences of their environment. The experimental basis for this hypothesis, however, is insufficient in view of the finding of White and White (17, 18) that growth inhibition is a general feature of all aromatic hydrocarbons which deprive the body of its cystine content by combining with it to form mercapturic acids or their degradation products. The difference between tumor-producing and inactive compounds is rather of a quantitative character.

Many workers believe that the carcinogens possess some peculiar chemical reactivity which is responsible for their specific influence on the living cell. Fieser, especially, has sought such a peculiarity by coupling polycyclic hydrocarbons with certain diazo compounds (6) or by oxidizing them with lead tetraacetate (7, 8). Recently he has expressed this idea in the more precise form that their peculiar reactivity towards sulfur-containing reagents enables them to initiate *in vivo* a chain reaction, whereby the carcinogens themselves form a new C–S bond (19). However, none of these reactions is highly specific for the carcinogenic hydrocarbons.

In contrast to these theories, it should be emphasized that the typical and most potent carcinogens are characterized by their chemical inertness and stability. However, among the numerous experiments carried out in this field some curious facts are found which, as we believe, permit one to draw some general conclusions about the principles determining carcinogenic activity.

Introduction of Side Chains

Alkyl substituents show a different influence on the activity of the parent ring structure according to their position. From the results, part of which are summarized in Tables I to III, it is obvious that in most cases a relation exists between certain positions of alkyl substitution and additional rings linked to the basal skeleton at the same points. Indeed, such a connection has already been accepted in some cases as a guide to the detection of new carcinogens (*e.g.*, 12).

Table I: Derivatives of 1,2-Benzanthracene

Methyl groups at	Activity	Corresponding cyclic product
3	0	
4	+	4,10-Dimethylene-1,2-benzanthracene
5	++	
6	++	1,2,5,6-Dibenzanthracene
5,6	+++	
2',6	0	
3',6	0	3'-Methyl-1,2,5,6-dibenzanthracene
7	?	
8	0	1,2,7,8-Dibenzanthracene
9	+++	3,4-Benzpyrene
10	+++	Cholanthrene
9,10	++++	
5,9	+++	4',5-Dimethylene-3,4-benzpyrene
5,10	++++	Cholanthrene
5,9,10	++++	4',5-Dimethylene-3,4-benzpyrene
6,7	?	1,2-Benznaphthacene
1'	0	
1',10	0	
2'	0	
3'	0	
2',7	0	
3',7	0	

Table II: Derivatives of 2,4-Dibenzphenanthrene

Methyl groups at	Activity	Corresponding cyclic product
1	+++	
2	++++	1,2,3,4-Dibenzphenanthrene
6	+	
7	?	
8	0	3,4,7,8-Dibenzphenanthrene
6,7	0	

Table III: Derivatives of 3,4-Benzpyrene

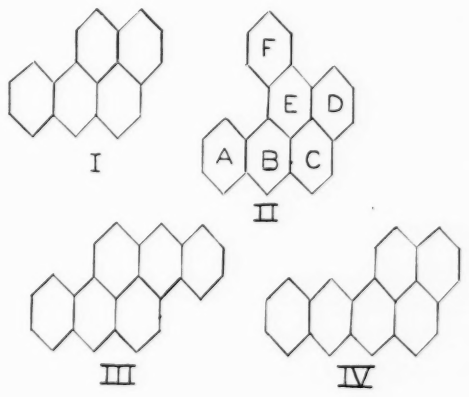
Methyl groups at	Activity	Corresponding cyclic product
2'	0	
3'	0	3,4-Naphthopyrene
4'	++	
5	++++	4',5-Dimethylene-3,4-benzpyrene
6	++	
9	++++	3,4,8,9-Dibenzpyrene

It can also be seen that methyl groups sometimes impair the activity of the basal compounds. In general, we can establish 3 categories of methyl groups with regard to their position: (a) methyl groups which enhance tumor production; (b) methyl groups which inhibit tumor production; (c) methyl groups without influence on tumor production. We can now establish the rule that *side chains produce active carcinogens if they imitate rings which enhance tumor production,* and *vice versa.* On the contrary, polar groups mostly diminish or destroy activity, even at positions where methyl groups exert a strong positive influence.

The "Active Principle" of Carcinogenic Compounds

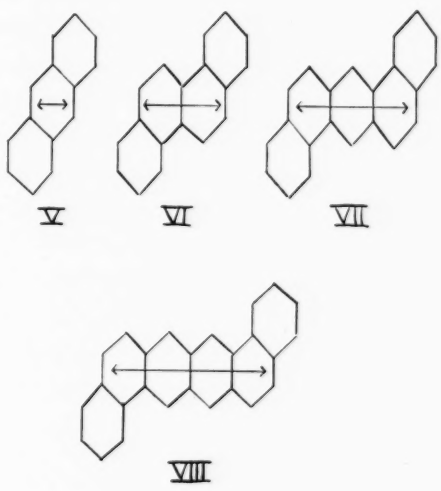
It can be concluded that a molecule is active only if it does not contain any point of pronounced reactivity. The molecule is active as a whole; *i.e., its shape and size determine its activity.* In this connection, it is of interest that in heterocyclic compounds also, similar forms of the molecules are required for carcinogenic activity as in polycyclic hydrocarbons. The correspondence between different series of substances indicates that all carcinogenic hydrocarbons and related compounds attack the same point inside the living cell; *viz., they are adsorbed by one and the same acceptor.* The proper adsorption of a molecule is the first condition for its activity.

The experiments of Hieger (13), with their suggestion that carcinogens are mutually replaceable, strongly support this hypothesis and urgently require extension to other groups of substances, of even more different structure than benzpyrene and 1,2,5,6-dibenzanthracene. At first sight it may appear difficult to imagine an acceptor which, for instance, should fit derivatives of 1,2-benzanthracene and 3,4-benzphenanthrene at the same time. However, from the experiments of Bachmann (1) on benzpyrene (formula I) it is evident that



the addition of further rings to a highly active structure, as in (II) or (III), again may lead to strongly active compounds. Therefore formula I can be considered as a part of II or III, and in the same way it is conceivable that all known carcinogens represent parts of the "ideal carcinogenic structure," which presumably would exhibit maximum activity because of its complete adaptation to the supposed acceptor. For example it can be seen that formula II includes the molecules of benzpyrene, 3,4-benzphenanthrene (rings ABEF), 1,2,3,4-dibenzphenanthrene (rings ABDEF), and chrysene (rings ABED).

The supposed acceptor clearly possesses a definite form and size, which sets an upper limit for the area to be covered by a carcinogen. It is known that a structure containing the naphthacene nucleus is inactive, although part of the molecule may represent a strongly active compound (*e.g.,* IV). A lower limit is given by the fact that the compounds become less easily adsorbed the smaller the number of their rings.[1] If one compares the compounds V through VIII from this point of view, it is seen that by lengthening the central axis of the basal form (V) a maximum of activity is reached by substance VII, and it seems desirable to synthesize compound VIII, which should be devoid of activity.

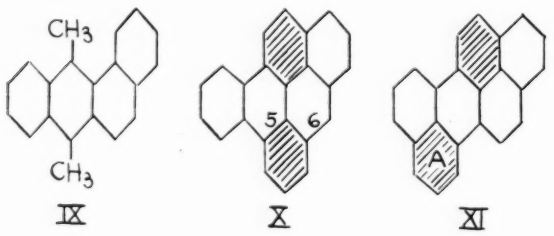


Some Conclusions from the Foregoing Statements

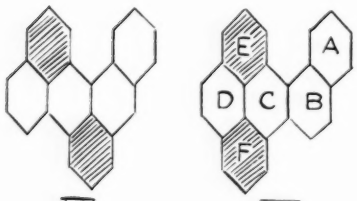
If we know at which positions aliphatic side chains enhance carcinogenic activity, we can construct new active cyclic structures and *vice versa.* But it must be borne in mind that a methyl group can give rise to 2 neighboring rings, and that only 2 ortho-methyl groups determine unambiguously the position of a new ring. This may be exemplified in the following case:

[1] In this respect, a direct comparison is possible with the formation of picrates. If only the monopicrates are compared, their dissociability is lowered and their color is intensified by increasing the number of aromatic rings.

For the highly potent 9,10-dimethyl-1,2-benzanthracene (IX) 4 corresponding cyclic structures can be constructed (X through XIII). A compound analogous to XI but containing a 5-membered ring in place of A has

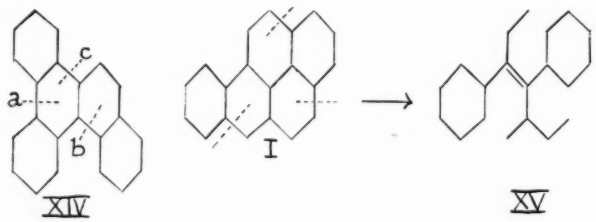


IX X XI



XII XIII

been described recently by Bachmann and Carmack (2). Structure XIII bears a close relationship to 1,2,3,4-dibenzphenanthrene (rings ABCEF) and structure X is an homologue of 5,6-dimethyl-3,4-benzpyrene. None of the 4 possibilities can be excluded *a priori*.

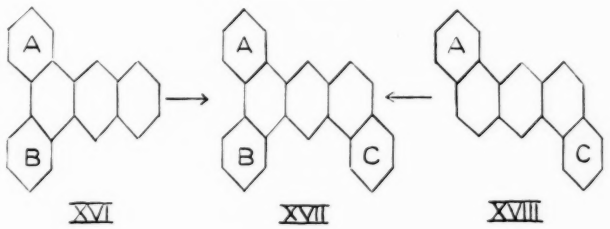


XIV XV

In the same way, known active polycyclic systems can be opened at one or more links to yield simple "open chain" structures. If, for example, we take 1,2,3,4-dibenzphenanthrene (XIV), then line (a) breaks the molecule up to 1,2-diphenylnaphthalene, line (b) to *cis*-9-styrylphenanthrene, and line (c) to *o*-(*α*-naphthyl)-diphenyl. It has recently been discovered by Dodds and his co-workers (3) that benzpyrene may be opened at 3 places at once to yield the hydrocarbon XV, a strong carcinogen, and a similar idea underlies the synthesis of 1-(*β*-styryl)-acenaphthene, an open-chain analogue of cholanthrene (11).

Restrictions of the Foregoing Rules

A more intimate consideration of the known facts leads to the conclusion that the geometrical conformity of carcinogenic agent and acceptor is a necessary but not sufficient condition. Tribenzanthracene (XVII) is inactive, although it contains the "active structure" of 1,2,5,6-dibenzanthracene (XVIII). It can be assumed that the additional ring B in XVII prevents adsorption on the surface of the acceptor, as in XVI. But such an



XVI XVII XVIII

effect would be hardly understandable, and it is more conceivable that tribenzanthracene is indeed adsorbed, not, however, in the desired direction (by rings A and C) but by rings A and B. We may formulate this hypothesis in the following way: *Molecules which represent a portion of the "ideal carcinogen" can be inactivated by additions which hinder their proper adsorption.* Obviously this assumption contains an explanation for the inhibiting influence of certain methyl groups mentioned above. It should be possible to test the hypothesis by the simultaneous application of pairs like 1,2,3,4- and 1,2,5,6-dibenzanthracene, which because of their equal molecular weight and their comparable solubility should have about an equal chance to reach the cell acceptor. Among these two partners the inactive compound will be the more readily adsorbed, as has been learned from the example of tribenzanthracene, and should impair or prevent the action of its blastomatogenic companion. Experiments in this direction are now at hand.

Final Remarks

(a) It is difficult to say at present why of two adsorbed molecules one is active and the other not. It may be that by proper adsorption some active points of the molecules are exposed, so as to induce (catalytically) a chain reaction without being attacked themselves, and that an unfit adsorption inactivates these points. But it appears possible also that chemical carcinogenesis is really the prevention of a normal process. It should be pointed out that all blastomatogenic processes appear to follow the same mechanism. This seems probable from the experiments of Morton (15) and of Engelbreth-Holm (4, 5) on the enhancing effect of carcinogenic agents on the occurrence of tumors in hereditarily predisposed mouse strains. It can be assumed, therefore, that carcinogenic compounds disturb cell division by mechanically burdening some part of the division apparatus and produce in this way the same effect as x-rays or spontaneous cancerization.

(b) It should be remarked that geometrical conditions similar to those for carcinogenic compounds are valid also for other groups of biological activity. On

this basis the synthetic stilbestrol has been developed from the given structure of estrone. An interesting relationship of this kind exists also between carboxylic and sulfonic acids (1, 16, 20) in that the latter are specific inhibitors of the action of the former.

Finally, it should be pointed out that the present hypothetical considerations are published before the necessary chemical and biological tests have been achieved, because under the present circumstances these may take a long time.

SUMMARY

The following principles for tumor production by chemical agents are hypothetically established:

1. The form and size of a carcinogenic hydrocarbon and of its related heterocyclic compounds determine its activity. Every substance which imitates more or less closely a given parent structure resembles it also in its blastomatogenic action.

2. In the living cell, the carcinogens are adsorbed by an acceptor possessing a definite adsorption area. This sets an upper limit for the dimensions of the active compounds. A lower limit is given by a decrease in adsorbability with decreasing size of the molecule.

3. All carcinogens are conceived as parts of an "ideal carcinogenic structure."

4. A carcinogen can be inactivated by additions which prevent its proper adsorption. The mechanism of this hindering effect is discussed.

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Abstracts

Reports of Experimental Research

Carcinogenic Compounds

DICKENS, F., and WEIL-MALHERBE, H. [Royal Victoria Infirmary, Newcastle-upon-Tyne, England] FACTORS AFFECTING CARCINOGENESIS. I. THE EFFECT OF LIPOID SOLVENTS ON TUMOR PRODUCTION BY 3,4-BENZPYRENE. Cancer Research, 2:560-566. 1942.

The subcutaneous injection into mice of solutions of benzpyrene in sesame oil or in arachis oil gave rise to tumors in a large proportion of the animals. The same dose of benzpyrene dissolved in either the liquid or the solid fraction of mouse fat, which had been purified and freed from phospholipins, produced only a few tumors. This result confirms and extends those of Peacock and Beck and Morton and Mider. Possible reasons for different results obtained by others are discussed.

It is not necessary for the suppression of carcinogenic activity that the lipoid material should be of homologous origin. A mixture of fats and lipins obtained from ox brain was equally effective.

Conclusive results could not be obtained with the ox liver extract or with the mouse lipins employed, owing to the prevalence of ulceration and consequent leakage of the injected material. Examination of the mouse in the ultraviolet beam is usually a valuable aid towards detection of such leakage, but when mouse fat was the solvent the apparently intact skin also fluoresced.

In such experiments the amount of carcinogen should not be too great, or any inhibitory tendency may be masked by its excessive activity.—Authors' summary.

LAW, L. W. [Roscoe B. Jackson Memorial Lab., Bar Harbor, Maine] MULTIPLE SKIN TUMORS IN MICE FOLLOWING A SINGLE PAINTING WITH 9:10 DIMETHYL—1:2 BENZANTHRACENE. Am. J. Path., 17:827-831. 1941.

Twenty-five papillomas appeared in 10 mice of the C57 brown strain following a single dorsal application of 0.3% 9:10 dimethyl—1:2 benzanthracene in benzene. Thirteen of the papillomas became transformed into carcinomas, 7 regressed completely, and 5 persisted as such without much apparent change.

Twenty-two of the growths appeared outside, but near, the painted area. Histological and biopsy studies were made of all abnormal growths. Two of the carcinomas were transplantable to mice of the same strain.

In contrast, 30 mice of the same strain painted twice weekly for 12 weeks with the same carcinogen developed multiple persisting papillomas and carcinomas wholly within the painted area.—L. W. L.

Hormones

GROSS, L. [Inst. for Med. Research, Christ Hosp., Cincinnati, Ohio] SPONTANEOUS RECOVERY FROM SARCOMA IN CASTRATED ADULT AND IN SEXUALLY IMMATURE MICE. Cancer Research, 2:571-575. 1942.

The resistance of castrated and of sexually immature suckling and weaned mice of the albino ES strain to sarcoma 37 was determined by the incidence of spontaneous regression of tumors produced by intradermal inoculation of 0.02 to 0.03 cc. of a 20% tumor cell suspension.

Of 172 castrated males, 27% recovered from the cutaneous tumors as compared with 14% of 179 normal control males. The incidence of spontaneous recovery in 186 castrated females was 24% as compared with 42% in 130 normal control females. Thus castration, performed either before or shortly after the onset of sexual maturity, increased the resistance of males to subsequent intradermal inoculation of sarcoma and decreased that of females.

Immature suckling male and female mice, inoculated at the age of 5 to 18 days, demonstrated a high resistance to sarcoma, 44% of 165 immature males and 39% of 139 immature females recovering from tumors. In the corresponding control experiments 12% of 160 adult males and 37% of 151 adult females recovered.

Sexually immature males and females, inoculated at the age of 16 to 26 days, immediately or shortly after weaning, had a low resistance to sarcoma since only 12% of 137 immature weaned males and 14% of 116 immature weaned females recovered from tumors, as compared with 13% of 101 control adult males and 44% of 91 control adult females.—Author's abstract.

LAW, L. W. [Roscoe B. Jackson Mem. Lab., Bar Harbor, Maine] EFFECT OF PSEUDOPREGNANCY ON MAMMARY CARCINOMA INCIDENCE IN MICE OF THE A STOCK. Proc. Soc. Exper. Biol. & Med., 48:486-487. 1941.

A definite increase in the incidence of mammary carcinoma occurred in females of a selected subline (F_{38} to F_{58} generations) of the inbred A strain of mice. Eleven, or 25.6%, of 43 experimental mice developed breast adenocarcinomas at an average age of 14.6 months compared with an incidence of 4.9% for control mice.—L. W. L.

Genetics

BITTNER, J. J. [Roscoe B. Jackson Mem. Lab., Bar Harbor, Maine] OBSERVATIONS ON THE GENETICS OF SUSCEPTIBILITY FOR THE DEVELOPMENT OF MAMMARY CANCER IN MICE. Cancer Research, 2:540-545. 1942.

Hybrids and backcross generation animals were obtained by reciprocal matings between mice of the high cancer

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A strain and the low cancer C57 black (B) strain. The observations on the incidence of breast carcinoma are in accord with the mendelian theory that susceptibility to breast cancer may be a single dominant factor.

There is some evidence that the influence transferred in the milk of females of the susceptible strain may be more concentrated after the mice have given birth to 3 or more litters.

Approximately 2,600 mice of the various hybrid generations were observed.—Author's abstract.

FEKETE, E., and LITTLE, C. C. [Roscoe B. Jackson Mem. Lab., Bar Harbor, Maine] OBSERVATIONS ON THE MAMMARY TUMOR INCIDENCE OF MICE BORN FROM TRANSFERRED OVA. Cancer Research, 2:525-530. 1942.

Fertilized ova of the C57 black low mammary tumor strain of mice were transferred, 52 hours after observation of a vaginal plug, into the uteri of mice belonging to the dba high mammary tumor strain, and *vice versa*. The mice born from transferred ova were nursed by mothers which gave birth to them. In addition to the foster nursing influence, these mice were also subjected to the intrauterine environment of their foster mothers.

In the transferred-ova C57 black mice mammary carcinomas occurred in 50% of the breeding females, while none of the transferred-ova dba animals developed them. This increase and decrease in mammary tumor incidence is greater than has been accomplished by foster nursing alone in these 2 strains.

It is possible that the intrauterine influence is at least partially responsible for the changes in both increasing and decreasing directions.

In the descendants of the original transferred-ova C57 black mice (1st, 2nd, and 3rd generations) the mammary tumor incidence was 73%.

The descendants of the original transferred-ova dba mice did not remain free from mammary growths. In the 1st, 2nd, and 3rd generations the incidence was 11.7%.

At the present time neither group of descendants can be considered as significantly different from its respective group of original animals obtained from transferred ova.—Authors' abstract.

STRONG, L. C. [Yale Univ. Sch. of Med., New Haven, Conn.] THE ORIGIN OF SOME INBRED MICE. Cancer Research, 2:531-539. 1942.

Data are presented showing the origin of 11 inbred strains of mice which are now being used in cancer and other biological research. Among these strains the A, C3H, CBA, and I are well known, as indicated partially by the analysis of the use of mice in Hartwell's monograph on carcinogenesis. The others, the CHI, C12I, C, JK, F, L, and N have also been inbred for at least 17 years. It is shown that the A, C3H, CBA, CHI, C12I, and C are somewhat genetically related although they have been separated from each other by 20 years of inbreeding. The other strains are not related to any of these or to each other. A representative survey of the contributions that have been made possible, in part, by the use of these strains is also included.—Author's abstract.

Tumors Induced by Radiation

HUEPER, W. C. [Warner Inst. for Therapeutic Research, New York, N. Y.] MORPHOLOGICAL ASPECTS OF EXPERIMENTAL ACTINIC AND ARSENIC CARCINOMAS IN THE SKIN OF RATS. Cancer Research, 2:551-559. 1942.

In experiments with congenitally hairless rats and their haired litter mates it was shown that the epidermis of this animal is capable of producing under proper stimulation (ultraviolet rays, arsenic) as great a variety of morphologically different carcinomas as that seen in the human skin. Indeed, the epidermal tumors obtained in the skin of the rat following an exposure to ultraviolet rays are even more complex than those occurring in man, for not only are they multiple, as a rule, but also are not infrequently associated with hyperplastic or sarcomatous proliferations of the mesenchymatous elements of the cutis. Carcinosarcomatous collision tumors are for this reason relatively common. The average life span of the rat is apparently shorter, in general, than the latent period of experimental arsenic carcinoma.—Author's abstract.

Immunology

APPEL, M., SAPHIR, O., JANOTA, M., and STRAUSS, A. A. [Michael Reese Hosp., Chicago, Ill.] COMPLEMENT-FIXING ANTIBODIES (BROWN-PEARCE CARCINOMA) IN THE BLOOD SERUM AND IN THE AQUEOUS FLUID OF THE ANTERIOR CHAMBER OF THE EYE. Cancer Research, 2:576-578. 1942.

Immunization of rabbits to the Brown-Pearce carcinoma does not confer protection against growth of this tumor in the anterior chamber of the eye. Complement fixation tests made on the blood serum and the aqueous humor reveal that while complement-fixing antibodies are present in high titer in the blood serum of tumor-bearing and immune rabbits, these antibodies do not gain access to the aqueous fluid. It is suggested that the barrier which exists between the blood and the aqueous humor, preventing the passage of antibodies from the former into the latter, is responsible for the successful growth of the Brown-Pearce carcinoma in the anterior chambers in tumor-immune rabbits when it will not grow in any other transplantation site.

Examination of the re-formed aqueous fluid which fills the anterior chamber after puncture revealed the presence of complement-fixing antibodies in this fluid to a degree approximating that found in the blood serum. This is believed to be due to an alteration in the permeability of the capillaries of the eye resulting from the disturbance caused by preliminary evacuation of the aqueous fluid.—Authors' abstract.

KHANOLKAR, V. R., and CHITRE, R. G. [Tata Memorial Hosp., Bombay, India] STUDIES IN ESTERASE (BUTYRIC) ACTIVITY. I. ESTERASE CONTENT OF SERUM OF MICE FROM CERTAIN CANCER-RESISTANT AND CANCER-SUSCEPTIBLE STRAINS. Cancer Research, 2:567-570. 1942.

The esterase activity in the serum of 2 strains of cancer-susceptible mice, A and C3H, and one strain of cancer-resistant, C57 black, has been determined.

It was found that the activity in the cancer-susceptible strains (36.2 ± 0.59 and 35.9 ± 0.94) was higher than that

in the cancer-resistant strain (16.6 ± 0.39). The difference is statistically significant.

No difference was noticed in the serum esterase of the 2 age groups, and of the males and females of the same strain.

The possible significance of liver metabolism in determining the susceptibility towards cancer is discussed.—Authors' summary.

Leukemia

LAW, L. W. [Roscoe B. Jackson Mem. Lab., Bar Harbor, Maine] THE EFFECT OF FOSTER NURSING ON THE GROWTH OF A TRANSMISSIBLE LEUKEMIA IN MICE. Science, 93:381-382. 1941.

Foster nursing of refractory mice of the C57 black and dba subline 1 mice by susceptible females of the dba subline 212 strain rendered the refractory mice susceptible to leukemic cells of a transmissible leukemia, line LL449.

The clinical course of the disease, as determined by infiltration of leukemic cells, blood counts, and length of life following inoculation, was similar in the refractory fostered mice and the susceptible mice.—L. W. L.

Treatment—Radiation, Chemotherapy, etc.

PLAUT, J. A., TENNANT, R., and OUGHTERSON, A. W. [Yale Univ. Sch. of Med., New Haven, Conn.] THE IRRADIATION OF TRANSPLANTED BAGG-JACKSEN AND YALE CARCINOMAS IN MICE, AS AFFECTED BY DIET AND FOSTER NURSING. Cancer Research, 2:546-550. 1942.

Neither diet nor foster nursing had any effect upon the radiosensitivity of the 2 tumors employed. The hosts were F_1 generation hybrids obtained by crossing C57 black, A, and Y strains. The diets were 2 commonly used commercial animal foods.

The tumors behaved differently with respect to each other, but had the same response in general to diet and host differences.—Authors' abstract.

Clinical and Pathological Reports

Ovary

PULSKAMP, M. H., GRISWOLD, R. A., and BLAIR, F. L. [Univ. of Louisville Sch. of Med., Louisville, Ky.] SECONDARY INTRARECTAL SOLID TERATOMA OF THE OVARY. South. Surgeon, 10:880-885. 1941.

A teratoma of the ovary, which had eroded through the anterior wall of the rectum and protruded from the anus, was extirpated successfully through an abdominal incision.—M. J. E.

SHANDS, H. R., and CLARK, R. L., Jr. [Jackson, Miss.] CARCINOMA OF THE OVARY. South. Surgeon, 10:813-818. 1941.

Of 250 patients with ovarian tumors 29 had malignant types. The astonishing results achieved occasionally in patients with apparently hopelessly advanced cancers is illustrated by a detailed case report. Complete resection of an adenocarcinoma had been impossible because of extensive pelvic metastases. Following the application of 50 mgm. of radium for 24 hours, roentgen therapy was administered. No evidence of tumor was discernible 5 years later.—M. J. E.

Gastrointestinal Tract

ABRAMS, M. J. [Brewster, Ala.], and TURBERVILLE, J. S. [Century, Fla.] LIPOSARCOMA OF THE STOMACH: REPORT OF A CASE. South. Surgeon, 10:891-896. 1941.

The patient died of omental and hepatic metastases from a liposarcoma of the stomach. Clinically the course of the disease had been indistinguishable from that commonly associated with carcinoma.—M. J. E.

BRINDLEY, G. V. [Scott and White Clinic, Temple, Texas] CARCINOMA OF THE COLON. FACTORS INFLUENCING PROGNOSIS. South. M. J., 35:171-180. 1942.

In 190 patients with cancer of the colon, removal of the tumor was possible in 66.6%. Of the patients operated upon, 71% lived more than 3 years and 62% more than 5 years. Of the latter, 10% will probably die later from recurrence.—H. G. W.

CLERF, L. H., and WIRTS, C. W., Jr. [Jefferson Hosp., Philadelphia, Pa.] THE IMPORTANCE OF GASTROSCOPY IN DIFFERENTIATING GASTRIC ULCER AND CARCINOMA. Rev. Gastroenterol., 9:1-7. 1942.

The gastroscopic and x-ray findings in 34 selected cases of gastric lesions are reported. The value of gastroscopy as an adjunct to the x-ray is indicated.—H. G. W.

CONLAN, F. [Pittston, Pa.] THE MALIGNANCIES OF THE RECTUM AND ANUS. Rev. Gastroenterol., 9:66-71. 1942.

General discussion.—H. G. W.

GARLOCK, J. H. [Mt. Sinai Hosp., New York, N. Y.] RADICAL SURGICAL TREATMENT FOR CARCINOMA OF THE CARDIAC END OF THE STOMACH. Surg., Gynec. & Obst., 74:555-560. 1942.

A study of 25 cases of which 9 were found operable. Four patients were alive without evidence of recurrence after 7 to 18 months.—H. G. W.

GRAY, H. K. [Mayo Clinic, Rochester, Minn.] THE DIAGNOSIS AND TREATMENT OF CANCER OF THE STOMACH. Surg., Gynec. & Obst., 74:487-490. 1942.

In the past 15 years there has been little improvement in the ratio of patients operated on to the total number found to have cancer of the stomach.—H. G. W.

GREENFIELD, I. [Kings County Hosp., Brooklyn, N. Y.] UNUSUAL METASTASIS OF RECTAL CARCINOMA. Am. J. Surg., 55:590-593. 1942.

A case of carcinoma of the rectosigmoid with metastasis to the left supraclavicular space (Virchow's lymph node) is reported.—H. G. W.

GROSS, P., and FREEDMAN, L. J. [Western Pennsylvania Hosp., Pittsburgh, Pa.] OBSTRUCTING SECONDARY CARCINOMA OF THE ESOPHAGUS. Arch. Path., 33:361-364. 1942.

Report of a case of obstructing carcinoma of the esophagus secondary to a clinically silent prostatic carcinoma.—H. G. W.

KOENIG, E. C., and CULVER, G. J. [Buffalo Gen. Hosp., Buffalo, N. Y.] REPORT OF TWO CASES OF CARCINOMA OF THE JEJUNUM. Radiology, 37:357-360. 1941.

Two cases of carcinoma of the jejunum are reported one of which was diagnosed roentgenographically.—C. E. D.

LAHEY, F. H. [Lahey Clinic, Boston, Mass.] ESOPHAGEAL TUMOR: REPORT OF A CASE. Lahey Clin. Bull., 2:194-197. 1942.

The surgical removal of a fibroma from the wall of the esophagus is reported. Differential diagnosis between intrathoracic goiter and intrinsic tumor of the esophagus was correctly settled at operation as soon as the inferior thyroid artery and recurrent laryngeal nerve were found running anterior to the tumor.—C. E. D.

LEMON, R. G., and BRODERS, A. G. [Mayo Clinic, Rochester, Minn.] A CLINICAL AND PATHOLOGICAL STUDY OF LEIOMYOSARCOMA, HEMANGIOENDOTHELIOMA OR ANGIOSARCOMA, AND FIBROSARCOMA OF THE STOMACH. Surg., Gynec. & Obst., 74:671-680. 1942.

A study of 14 cases which included 11 leiomyosarcomas, 2 hemangioendotheliomas, and 1 fibrosarcoma, with emphasis on their lesser frequency and malignancy as compared with lymphosarcomas. These tumors can be treated only by operation, in contrast to the lymphosarcomas which are radiosensitive. Of the 14 patients, 6 are still alive after an average postoperative interval of 4 years.—H. G. W.

McKITTRICK, L. S. [Massachusetts Gen. Hosp., Boston, Mass.] CARCINOMA OF THE RIGHT COLON. ITS DIAGNOSIS AND PRINCIPLES OF TREATMENT. Rhode Island M. J., 24:161-165. 1941.

In surgery of cancer of the right colon, because of the decreased operative risk and the greater possibility of performing a radical resection in otherwise inoperable cases, the author advocates a 2-stage procedure consisting in anastomosis of the ileum and transverse colon, followed, 3 weeks later, by a resection of the involved bowel.—M. J. E.

NIELSEN, J. [Radiumstation, Copenhagen, Denmark] KLINISCHE VERSUCHE ZUR STRAHLENBEHANDLUNG DES SPEISERÖHRENKREBSES. [RADIOTHERAPY OF ESOPHAGEAL CANCER.] Acta radiol., 21:352-367. 1940.

In most cases of cancer of the esophagus radiotherapy is administered only for its palliative action. Prior to 1931, 36 patients received radium or roentgen therapy, at times combined with a gastrostomy, and the average survival was 4½ months. Symptomatic roentgen irradiation has been more energetically employed since 1931, and the 89 patients treated in the latter period, who received an estimated tumor dose of 1,500 to 2,000 r, survived 5 to 7 months. In 17 additional persons the condition appeared sufficiently favorable to attempt a cure by protracted fractional irradiation. The dose administered to the tumor in this series varied from 4,000 to 4,500 r. Three patients were alive after 2½ to 3½ years, while the average time of survival of the remainder was 12 months. An example of a cure of cancer of the hypopharynx, achieved by roentgen therapy, is described. This patient was symptom-free 6 years after the conclusion of treatment.—M. J. E.

RITTER, H. H., and SHAFFER, J. M. [New York Post-Graduate Med. Sch., New York, N. Y.] LYMPHOSARCOMA OF THE INTESTINAL TRACT. Am. J. Surg., 55:611-612. 1942.

Report of a case with apparent 5 year cure following surgical removal.—H. G. W.

ROSSER, C. [Dallas, Tex.] THE EXTERIORIZATION OF COLONIC CANCERS. South. Surgeon, 10:874-879. 1941.

Of 50 patients with cancer of the colon 31 were considered to have operable neoplasms, and 45% of these survived 2 to 5 years after operation. The author favors resection in several stages, chiefly to relieve manifestations of obstruction. The initial procedure advocated is a Mikulicz type of colostomy with an afferent and efferent loop in the appropriate segment of the bowel.—M. J. E.

ROSSER, C. [Baylor Univ. Coll. of Med., Dallas, Tex.] THE SURGICAL MANAGEMENT OF COLONIC CANCER. South. M. J., 35:262-267. 1942.

An analysis of 50 cases of cancer of the colon, emphasizing the better prognosis than in cancer of the rectum and rectosigmoid.—H. G. W.

SANDERS, R. L. [Memphis, Tenn.] MULTIPLE DISSIMILAR CARCINOMAS OF THE STOMACH. South. Surgeon, 10:695-704. 1941.

Preoperative roentgen evidence of 2 distinct gastric cancers was confirmed at operation: a colloid carcinoma involved the cardia, a medullary cancer the pyloric region. Both metastasized to regional nodes, but the patient had no evidence of a recurrence 14 months later. Illustrations are included.—M. J. E.

WHITE, R. J. [Fort Worth, Tex.] HEMANGIOMA OF THE TERMINAL ILEUM WITH RECURRENT HEMORRHAGES FROM THE RECTUM. South. Surgeon, 10:886-890. 1941.

The difficulties in diagnosis presented by the symptom, rectal bleeding, are illustrated by a case report. The involved intestine was resected.—M. J. E.

Book Review

OCCUPATIONAL TUMORS AND ALLIED DISEASES. By W. C. Hueper, M. D. Charles C. Thomas, Springfield, Illinois. 1942. XXVIII + 896 pages. Price $8.00.

In the preface it is stated that the chief aim of this book is to furnish an extensive source of information concerning malignant diseases of occupational origin, and it may be said at once that the author has admirably succeeded.

But the volume is much more than a mere catalogue of occupational neoplasms. These are compared wherever possible with experimentally produced tumors and the data concerning both are correlated and critically analyzed.

The arrangement of the material is by organs and, as was to have been expected in an era when a formidable increase in cancer of the lung is so anxiously watched by both clinician and pathologist, the chapter on tumors of the respiratory system is by far the longest.

There can be no doubt of a general increase in the frequency of pulmonary neoplasms, but puzzling geographical variations appear in their incidence. Thus, for example, they are common in some highly industrialized districts of Germany and not in others. This observation, says the author, does not warrant the summary dismissal of industrialization as an etiological influence, for the existing knowledge of occupational carcinogenic agents is now sufficiently exact and extensive to emphasize the importance of specific agents as against the vague concept of nonspecific, chronic, irritating industrial factors.

In sifting the etiology of pulmonary carcinomas the author is inclined to discount antecedent infections like tuberculosis or influenza; dust as such, irrespective of its chemical character; silica dust; war gases and noxious fumes from factories, gasoline motors, and Diesel engines; and mechanical trauma to the chest. Among possible causes are listed the silicates contained in asbestos; iron dust; arsenic fumes; nickel; soot and tar, including dust from tarred roads; tobacco smoke. Chromates are mentioned as a probable cause, and radioactive substances as certainly provocative of cancer of the lung.

One of the closing chapters, on the cause of cancer in general, compares the various theories in the light of modern knowledge drawn from the study of induced neoplasia in animals and of industrial malignant disease. It is concluded that the allergic concept is most compatible with the known facts, for no other, with the possible exception of the virus hypothesis, can furnish a satisfactory explanation of the appearance of occupational neoplasms many years after cessation of exposure to the implicated noxae. An allergic theory possesses the advantage, moreover, that it can be coordinated with the virus hypothesis in a much more plausible way than any of the other explanations that have been advanced.

Whether the reviewer agrees with this point of view is neither here nor there. The book contains a prodigious amount of information on industrial cancer, and should be in the possession of all those to whom it is addressed: investigators of cancer, public health agencies and legislative bodies, workmen's compensation boards, lawyers, industrial managers and engineers, and life and accident insurance companies.

The last chapter contains a full discussion of whether or not a single trauma can elicit malignant disease, either directly or indirectly, in a previously healthy tissue. The former possibility is rejected once for all, and the latter contemplated with deep suspicion.

The number of misprints is remarkably small, a matter for congratulation of both author and publisher, but several curious little expressions suggest that English is not the author's mother tongue. Thus "the skin and its appendiceal organs" is employed for the skin and its appendages, though the customary phrase appears in the Table of Contents; "palpatory tenderness" for tenderness on palpation, and so on. Still, these little slips are not numerous and it is perhaps ungracious to mention them at all.

The bibliography contains a good thirty-five hundred entries.

WILLIAM H. WOGLOM